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(54) Title: **SURFACTANT PROTEIN D FOR THE PREVENTION AND DIAGNOSIS OF PULMONARY EMPHYSEMA**

(57) Abstract: Surfactant protein D (SP-D) is a 43-kDa member of the collectin family of collagenous lectin domain-containing proteins that is expressed in epithelial cells of the lung. The SP-D gene was targeted by homologous recombination in embryonic stem cells that were used to produce SP-D (-/-) mice. The SP-D (-/-) deficiency caused inflammation, increased oxidant production by isolated alveolar macrophages, abnormal surfactant structure and levels, and decreased SP-A expression. Therefore, disclosed is the SP-D (-/-) mouse as an excellent model for emphysema. Also included are models for testing emphysema therapies in the mouse model, methods for using SP-D protein or DNA as a treatment for emphysema and pulmonary infections, and diagnosis.

SURFACTANT PROTEIN D FOR THE PREVENTION AND DIAGNOSIS OF PULMONARY EMPHYSEMA**GOVERNMENT INTEREST IN THE INVENTION**

Certain aspects of the invention disclosed herein were made with United States government support under
5 National Institutes of Health grants HL 41320, SCOR HL 56387, HL 28623, HL 58795, and HL03905. The United
States government has certain rights in these aspects of the invention.

FIELD OF THE INVENTION

The present invention relates generally to the field of biologically active proteins. More specifically the
10 present invention relates to SP-D proteins involved in pulmonary surfactant homeostasis and structure, and alveolar
structure in the lungs and SP-D (-/-) null mice.

BACKGROUND OF THE INVENTION

Pulmonary surfactant is essential for normal lung mechanics and gas exchange in the lung. Pulmonary
15 surfactant is produced by type II epithelial cells and is made up of a phospholipid component which confers the ability
of surfactant to lower surface tension in the lung. In addition, there are proteins associated with the surfactant called
collectins which are collagenous, lectin domain-containing polypeptides. Two of these, designated surfactant protein
A (SP-A) and surfactant protein D (SP-D), are likely involved in surfactant structure and function and host defense.
Both quantitative and qualitative deficiencies in pulmonary surfactant are associated with neonatal respiratory
20 distress, adult respiratory distress syndrome, congenital deficiencies of surfactant protein B, and allergic asthma. In
addition, deficiency in pulmonary surfactant may contribute to the increased susceptibility of some individuals to
microbial challenge, especially in the setting of inadequate or impaired specific immunity. These disorders as well as
some disorders associated with increased risk of pneumonia (cystic fibrosis, asthma, prematurity, chronic bronchitis,
diffuse alveolar damage) may also be associated with acquired defects or deficiency in collectin function. Alveolar
25 surfactant pools are regulated at multiple levels including intracellular synthesis, secretion, re-uptake and degradation
of these components by alveolar macrophages. The synthesis and clearance of surfactant phospholipids and proteins
is further influenced by developmental, mechanical, and humoral stimuli that serve to maintain steady-state surfactant
concentrations after birth.

The role of the collectins in surfactant and normal lung function has been extensively investigated. The
30 collectin family of C-type lectins includes a number of molecules with known host defense functions. SP-A and SP-D,
also C-type lectins, bind influenza and herpes simplex viruses as well as gram positive and gram negative bacteria and
various fungi. By binding they enhance uptake by alveolar macrophages and neutrophils. Various cellular binding sites
for SP-A and SP-D have been identified on alveolar macrophages or, in the case of SP-A, on type II epithelial cells. The
critical role of SP-A in host defense was supported by the observation that SP-A-deficient mice are susceptible to
35 infections by group B streptococcus, *Pseudomonas aeruginosa*, Respiratory syncytial virus, adenovirus, and

mycoplasma *in vivo*. Thus, there is a clear role for SP-A and a likely role for SP-D in respiratory defense mechanisms. Collectins may also participate in the recognition or clearance of other complex organic materials, such as pollens and dust mite allergens. However, to date no human diseases have been associated with specific deficiencies in SP-A or SP-D.

5 SP-D is a 43 kilodalton protein that has been proposed to play a role in host defense in the lung. Its cDNA and gene have been sequenced in various mammals including humans. SP-D shares considerable structural homology with other C-type lectins, including surfactant protein A (SP-A), conglutinin, bovine collectin 43, and mannose binding protein. *In vitro* studies and its close structural relationship to a mammalian Ca^{2+} -dependent lectin family (particularly shared structural motifs) support its role in host defense. SP-D is synthesized primarily and at relatively
10 high concentrations by Type II epithelial cells and nonciliated bronchiolar epithelial cells in the lung but may also be expressed in the gastrointestinal tract, heart, kidney, pancreas, genitourinary tract and mesentery cells. *In vitro* studies demonstrated that SP-D binds to the surface of organisms via its lectin domain (or sugar binding domain) which leads to binding, aggregation, opsonization and, in some instances, activation of killing by phagocytes *in vitro*. SP-D binds to lipopolysaccharide, various bacteria, fungi and viruses, including influenza virus. It also binds to both
15 alveolar macrophages and polymorphonuclear cells. It may possibly play a role in surfactant phospholipid homeostasis, including the effects of SP-A on phospholipid metabolism by Type II cells *in vitro*, however, this is controversial and the precise role of SP-D *in vivo* is still unclear.

In vitro studies support the concept that surfactant proteins may be important in the regulation of surfactant homeostasis. Although the hydrophobic surfactant proteins SP-B and SP-C have roles in production of the surfactant
20 monolayer, *in vitro* studies indicated that surfactant protein A may also facilitate surfactant uptake and/or secretion by type II epithelial cells. In fact, it was widely believed that SP-A would have a major role in surfactant homeostasis. However, recent studies of SP-A null mice have not supported the primary role of surfactant protein A in surfactant secretion or re-uptake. The absence of SP-A does not lead to obvious physiologic or morphologic structural abnormalities of the lung. SP-A null mutant mice lack tubular myelin figures but produce highly functional surfactant
25 that absorbs rapidly and produces monolayers. Surfactant lipid synthesis, secretion, and re-uptake were essentially normal in SP-A null mice.

Therefore, the additional surfactant protein which acts in surfactant regulation is still not identified. In addition, the precise role of SP-D in normal lung function has not been clearly defined at this point and its role in disease or disease susceptibility is unclear.

30

SUMMARY OF THE INVENTION

The present invention provides an SP-D(-/-) mouse which can be used as a model for emphysema. Previously it was not known that SP-D protein was involved in lung lipid homeostasis. Nor was it known that an SP-D null mouse would have the symptoms of emphysema.

One embodiment of the invention is a non-human mammalian model for emphysema comprising an SP-D(-/-) non-human mammal.

A further embodiment is a method for the purification and treatment of pulmonary disease by introducing mammalian SP-D protein, or vectors expressing the mammalian SP-D protein into a human or mammal in an amount effective to reduce the symptoms of the disease or to prevent the disease.

A further embodiment is a pharmaceutical composition effective in treating pulmonary disease which is a mixture of SP-D protein with a pharmaceutically acceptable carrier.

A further embodiment is a biologically active agent for treating pulmonary disease in mammals which is an agent that up-regulates SP-D.

A further embodiment is a biologically active agent for treating pulmonary disease in mammals which is an agent that interacts with the SP-D protein.

A further embodiment is a method for diagnosing susceptibility to pulmonary disease in mammals by identifying a mutation in the SP-D gene which results in deficient SP-D, identifying that mutation in a test mammal by PCR, hybridization, or ELISA.

A further embodiment is a method of identifying pharmaceutical agents useful in treating pulmonary disease by allowing the SP-D null mouse to develop pulmonary disease, administering a pharmaceutical agent to the mammal, and identifying the agent as effective if the pulmonary disease improves.

A further embodiment is a method of purifying SP-D antibodies with a solid phase lung homogenate from any mouse which does not produce SP-D protein.

A further embodiment is a method for the prevention of pulmonary disease by introducing mammalian SP-D protein, or vectors expressing the mammalian SP-D protein into a human in an amount effective to reduce the symptoms of or prevent pulmonary disease, wherein the pulmonary disease is selected from the group consisting of: reactive oxygen mediated disease, chemically induced lung injury, injury due to oxygen radicals, injury due to ozone, injury due to chemotherapeutic agents, inflammatory and infectious diseases, reperfusion injury, drowning, transplantation, and rejection.

A further embodiment of the invention is a method for the treatment of viral disease by introducing mammalian SP-D protein, or vectors expressing the mammalian SP-D protein into a human in an amount effective to reduce the number of viruses or symptoms of the viral disease. Preferably, the viruses are adenovirus, RSV, and Influenza virus.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Comparison of changes in fractional areas (% Fx Area) of airspace (a) and respiratory parenchyma (b) with age in SP-D (-/-) mice and age-matched SP-D (+/+) controls. Analysis of changes in these parameters with age for each individual genotype (c and d). Data are expressed as % fractional area and represent the mean \pm SE.

Figure 2: Deflation limbs of pressure-volume curves from SP-D (+/+) and SP-D (-/-) mice. Data are expressed as ml/kg and represent the mean \pm SE.

Figure 3: Pro-inflammatory cytokines in lung homogenates from SP-D (-/-) mice. Concentrations of TNF- α , IL-1 β , IL-6 and MIP-2 were assessed in lung homogenates from SP-D (-/-) (solid bar) and SP-D (+/+) (hatched bar) mice. Data are expressed as pg/ml and represent the mean \pm SE with n=5 mice per group; *p<0.05 compared to SP-D (+/+) mice.

Figure 4: Hydrogen peroxide production in alveolar macrophages from SP-D (-/-) (solid bar) was assessed from 1×10^6 macrophages isolated from broncho alveolar lavage fluid (BALF) as compared to SP-D (+/+) mice (hatched bar) with and without PMA stimulation. Data are expressed as M of H₂O₂ and represent the mean \pm SE with n=4 mice per group; *p<0.05 compared to SP-D (+/+) mice.

Figure 5: Lung colony counts in SP-D(-/-) and SP-D(+/-) mice after infection with Gp B Streptococcus (GBS).

Figure 6: Lung colony counts in SP-D(-/-) and SP-D(+/-) mice after infection with *Haemophilus influenzae* (H. flu).

Figure 7: Total cell count in Bronchoalveolar lavage (BAL) fluid after infection with GBS and H. flu.

Figure 8: Cytokine levels in lung homogenates after infection with GBS and H.flu.

Figure 9: BAL nitrite levels after infection with GBS and H.flu.

Figure 10: Phagocytosis analyzed by light microscopy and FACS analysis after infection with GBS and H.flu.

Figure 11: Hydrogen peroxide and superoxide levels in macrophages isolated from BAL after infection with GBS and H.flu.

Figure 12: Effects of SP-D protein treatment on SP-D (-/-) mice.

Figure 13: Total lung and alveolar lavage clearance kinetics of SP-D protein in mice.

Figure 14: Adenoviral vector Ad-rSPD containing rat SP-D cDNA.

Figure 15: Quantification of immunoblots of SP-A and SP-D in alveolar washes from wild type and CCSP-IL-4 mice (IL-4 mice). p<0.01.

Figure 16: RSV and IAV titers were determined by quantitative plaque assays of lung homogenates. Viral titers of RSV were significantly greater 3 and 5 days after administration of 10^7 pfu RSV(Graph A) in SP-D -/- (open bar) compared to wild type (hatched bar) mice. Lung homogenate titers of IAV were significantly greater for SP-D -/- (open bar) compared to wild type (hatched bar) mice 3 and 5 days after infection (Graph B). Data are mean \pm SEM with n=15 mice per group (Graph A) and n=10 mice per group (Graph B). *p<0.05 compared to wild type mice.

Figure 17: Lung cells were recovered by bronchoalveolar lavage, stained with trypan blue and counted under light microscopy. SP-D -/- mice (open bar) had increased total cell counts in BAL fluid 3 and 5 days after RSV infection (graph A) compared to wild type mice (hatched bar). SP-D -/- (open bar) had increased total cell counts in BAL fluid 3 and 5 days after IAV infection (graph B). Data are mean \pm SEM with n=8 mice per group, *p<0.05 compared to wild type mice.

Figure 18: Cytospin preparations of bronchoalveolar lavage fluid were stained with DIFF-QUIK to identify macrophages, lymphocytes and polymorphonuclear leukocytes. The percentage of neutrophils in BAL fluid was significantly greater 3 and 5 days after administration of 10^7 pfu RSV to SP-D $-/-$ (open bar) compared to wild type (hatched bar) mice (Graph A). Similarly, the percentage of neutrophils in BAL fluid was significantly greater 3 and 5 days after administration of 10^5 pfu IAV to SP-D $-/-$ (open bar) mice compared to wild type (Graph B). Data are mean \pm SEM with n=8 mice per group, *p < 0.05 compared to wild type mice.

DETAILED DESCRIPTION OF THE INVENTION

The invention is related to a mouse model of emphysema which is SP-D $-/-$, a method for the prevention and treatment of emphysema with SP-D protein or nucleic acid, and methods of identifying pharmaceuticals useful for the treatment of emphysema and other lung diseases using the SP-D $-/-$ mouse model.

An SP-D $-/-$ knockout mouse was produced to identify the role of SP-D in normal lung function and development and to demonstrate the temporal progression of postnatal airspace enlargement and spontaneous inflammatory changes in the lungs of these mice. SP-D $-/-$ mice develop progressive pulmonary emphysema, associated with chronic inflammation and increased oxidant production by alveolar macrophages. The lung abnormalities make this mouse an excellent model for emphysema. Because there are very few existing therapies for treatment of emphysema, the most common being lung volume reduction surgery, the model is urgently needed. Based on the mouse model for emphysema, a number of ways to test SP-D protein and expression vectors, and potential pharmaceuticals in the mouse model for efficacy in treating emphysema or other forms of chronic lung injury are provided. The use of SP-D protein and expression vectors to treat various other diseases of aberrant surfactant production, pulmonary fibrosis, sarcoidosis, lung injury, toxicant/oxygen exposure, infection, increased oxidant exposure is also provided. Lastly, methods for using SP-D cDNA, SP-D antibodies, PCR, and differential hybridization techniques to identify patients at risk for emphysema, pulmonary distress syndromes, and other types of respiratory diseases are provided.

Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

As used herein, the term "pulmonary administration" refers to administration of a formulation of the invention through the lungs by inhalation.

As used herein, the term "inhalation" refers to intake of air to the alveoli. In specific examples, intake can occur by self-administration of a formulation of the invention while inhaling, or by administration via a respirator, e.g., to a patient on a respirator. The term "inhalation" used with respect to a formulation of the invention is synonymous with "pulmonary administration."

As used herein, the term "parenteral" refers to introduction of a SP-D into the body by other than the intestines, and in particular, intravenous (i.v.), intraarterial (i.a.), intraperitoneal (i.p.), intramuscular (i.m.), intraventricular, and subcutaneous (s.c.) routes.

As used herein, the term "aerosol" refers to suspension in the air. In particular, aerosol refers to the particulation of a formulation of the invention and its suspension in the air. According to the present invention, an aerosol formulation is a formulation comprising a SP-D that is suitable for aerosolization, i.e., particulation and suspension in the air, for inhalation or pulmonary administration.

5 As used herein, the term "systemic" refers to a disease or disorder, or original site of injury distant to the lung or involving the entire body of the organism. The term "local" therefore is used herein with respect to the lung.

For the sake of clarity, the present invention is described in detail in sections relating to SP-D, aerosol formulations, and methods for treatment and prophylaxis.

10 Pulmonary Delivery of SP-D Protein or nucleic acid

The present invention contemplates formulations comprising SP-D protein or nucleic acid for use in a wide variety of devices that are designed for the delivery of pharmaceutical compositions and therapeutic formulations to the respiratory tract. The preferred route of administration of the present invention is in the aerosol or inhaled form. The SP-D of the present invention, combined with a dispersing agent, or dispersant, can be administered in an aerosol
15 formulation as a dry powder or in a solution or suspension with a diluent.

As used herein, the term "dispersant" refers to a agent that assists aerosolization of the protein or absorption of the protein in lung tissue, or both. Preferably the dispersant is pharmaceutically acceptable. As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government as listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and
20 more particularly in humans. Suitable dispersing agents are well known in the art, and include but are not limited to surfactants and the like. For example, surfactants that are generally used in the art to reduce surface induced aggregation of the protein caused by atomization of the solution forming the liquid aerosol may be used. Nonlimiting examples of such surfactants are surfactants such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitan fatty acid esters. Amounts of surfactants used will vary, being generally within the range or
25 0.001 and 4% by weight of the formulation. In a specific aspect, the surfactant is polyoxyethylene sorbitan monooleate or sorbitan trioleate. Suitable surfactants are well known in the art, and can be selected on the basis of desired properties, depending on the specific formulation, concentration of SP-D, diluent (in a liquid formulation) or form of powder (in a dry powder formulation), etc.

Moreover, depending on the choice of the SP-D protein, peptide, or nucleic acid, the desired therapeutic
30 effect, the quality of the lung tissue (e.g., diseased or healthy lungs), and numerous other factors, the liquid or dry formulations can comprise additional components, as discussed further below.

The liquid aerosol formulations contain the SP-D protein or nucleic acid and a dispersing agent in a physiologically acceptable diluent. The dry powder aerosol formulations of the present invention consist of a finely divided solid form of the SP-D protein or nucleic acid and a dispersing agent. With either the liquid or dry powder
35 aerosol formulation, the formulation must be aerosolized. That is, it must be broken down into liquid or solid particles

in order to ensure that the aerosolized dose actually reaches the alveoli. In general the mass median dynamic diameter will be 5 micrometers or less in order to ensure that the drug particles reach the lung alveoli (Wearley, L. L., 1991, 1991, Crit. Rev. in Ther. Drug Carrier Systems 8:333). The term "aerosol particle" is used herein to describe the liquid or solid particle suitable for pulmonary administration, i.e., that will reach the alveoli. Other considerations such as construction of the delivery device, additional components in the formulation and particle characteristics are important. These aspects of pulmonary administration of a drug are well known in the art, and manipulation of formulations, aerosolization means and construction of a delivery device require at most routine experimentation by one of ordinary skill in the art.

Other advantageous carriers include aerodynamically light particles made of a biodegradable material and having a tap density of less than 0.4 g/cm³ and a mass mean diameter between 5 and 30 μ m. Examples of such particles are presented in Hanes, et al, U.S. patent No. 6,136,295, issued October 24, 2000. Typically the particles are formed of biodegradable polymers, for example, the particles may be formed of a functionalized polyester graft copolymer consisting of a linear alpha hydroxy acid polyester backbone having at least one amino acid group incorporated therein and at least one poly(amino acid) side chain extending from an amino acid group in the polyester backbone.

With regard to construction of the delivery device, any form of aerosolization known in the art, including but not limited to nebulization, atomization or pump aerosolization of a liquid formulation, and aerosolization of a dry powder formulation, can be used in the practice of the invention. A delivery device that is uniquely designed for administration of solid formulations is envisioned. Often, the aerosolization of a liquid or a dry powder formulation will require a propellant. The propellant may be any propellant generally used in the art. Specific nonlimiting examples of such useful propellants are a chlorofluorocarbon, a hydrofluorocarbon, a hydrochlorofluorocarbon, or a hydrocarbon, including trifluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof.

In a preferred aspect of the invention, the device for aerosolization is a metered dose inhaler. A metered dose inhaler provides a specific dosage when administered, rather than a variable dose depending on administration. Such a metered dose inhaler can be used with either a liquid or a dry powder aerosol formulation. Metered dose inhalers are well known in the art.

Once the SP-D protein or nucleic acid reaches the lung, a number of formulation-dependent factors effect the drug absorption. It will be appreciated that in treating an SP-D related disease or disorder that requires circulatory levels of the SP-D, such factors as aerosol particle size, aerosol particle shape, the presence or absence of infection, lung disease or emboli may affect the absorption of the protein. For each of the formulations described herein, certain lubricators, absorption enhancers, protein stabilizers or suspending agents may be appropriate. The choice of these additional agents will vary depending on the goal. It will be appreciated that in instances where local delivery of the SP-D protein or nucleic acid is desired or sought, such variables as absorption enhancement will be less critical.

In a further embodiment, an aerosol formulation of the present invention can include other active ingredients in addition to the SP-D protein or nucleic acid. In a preferred embodiment, such active ingredients are those used for the treatment of lung disorders. For example, such additional active ingredients include, but are not limited to, bronchodilators, antihistamines, epinephrine, and the like, which are useful in the treatment of asthma. In another embodiment, the additional active ingredient can be an antibiotic, e.g., for the treatment of pneumonia. In a preferred embodiment, the antibiotic is pentamidine.

In general, the SP-D protein or nucleic acid of the present invention, or the fragment or analog or derivative thereof is introduced into the subject in the aerosol form in an amount between 0.01 mg per kg body weight of the mammal up to about 100 mg per kg body weight of said mammal. In a specific embodiment, the dosage is dosage per day. One of ordinary skill in the art can readily determine a volume or weight of aerosol corresponding to this dosage based on the concentration of SP-D protein or nucleic acid in an aerosol formulation of the invention; alternatively, one can prepare an aerosol formulation which with the appropriate dosage of SP-D protein or nucleic acid in the volume to be administered, as is readily appreciated by one of ordinary skill in the art. It is also clear that the dosage will be higher in the case of inhalation therapy for a systemic disease or disorder involving SP-D, and lower for a lung disease or disorder involving SP-D, since the local concentration of SP-D protein or nucleic acid in the lung will be greater if the protein is administered to the lung. It is an advantage of the present invention that administration of SP-D protein or nucleic acid directly to the lung allows use of less SP-D protein or nucleic acid, thus limiting both cost and unwanted side effects.

The formulation may be administered in a single dose or in multiple doses depending on the disease indication. It will be appreciated by one of skill in the art the exact amount of prophylactic or therapeutic formulation to be used will depend on the stage and severity of the disease, the physical condition of the subject, and a number of other factors.

Systems of aerosol delivery, such as the pressurized metered dose inhaler and the dry powder inhaler are disclosed in Newman, S. P., Aerosols and the Lung, Clarke, S. W. and Davia, D. editors, pp. 197-22 and can be used in connection with the present invention.

It is particularly contemplated that a liposome formulation may be especially effective for administration of SP-D protein or nucleic acid by inhalation. This is particularly so where long term administration is desired (See Wearley, 1991, Crit. Rev. in Ther. Drug Carrier Systems 8:333).

30 Liquid Aerosol Formulations

The present invention provides aerosol formulations and dosage forms for use in treating subjects suffering from an SP-D related disease or disorder. In general such dosage forms contain one or more SP-D proteins or nucleic acids, or fragments, derivatives or analogs thereof in a pharmaceutically acceptable diluent. Pharmaceutically acceptable diluents include but are not limited to sterile water, saline, buffered saline, dextrose solution, and the like. In a specific embodiment, a diluent that may be used in the present invention or the pharmaceutical formulation of the

present invention is phosphate buffered saline, or a buffered saline solution generally between the pH 7.0-8.0 range, or water.

The liquid aerosol formulation of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, surfactants and excipients. Such carriers may serve simply as bulking agents when it is desired to reduce the SP-D protein or nucleic acid concentration in the powder or liquid which is being delivered to a patient, but may also serve to enhance the stability of the SP-D protein or nucleic acid composition and to improve the dispersability of the powder or liquid within a dispersion device in order to provide more efficient and reproducible delivery of the SP-D protein or nucleic acid and to improve handling characteristics of the protein or nucleic acid such as flowability and consistency to facilitate manufacturing and powder or liquid filling.

Suitable carrier materials may be in the form of an amorphous powder, a crystalline powder, a combination of amorphous and crystalline powders or a liquid. Suitable materials include carbohydrates, e.g., monosaccharides such as fructose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, cellobiose, and the like; cyclodextrins, such as 2-hydroxypropyl- β -cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; (b) amino acids, such as glycine, arginine, aspartic acid, glutamic acid, cysteine, lysine, and the like; (c) organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, magnesium gluconate, sodium gluconate, tromethamine hydrochloride, and the like; (d) peptides and proteins, such as aspartame, human serum albumin, gelatin, and the like; (e) alditols, such as mannitol, xylitol, and the like. A preferred group of carriers includes lactose, trehalose, raffinose, maltodextrins, glycine, sodium citrate, tromethamine hydrochloride, human serum albumin, and mannitol.

Such carrier materials may be combined with the SP-D protein or nucleic acid prior to administration, i.e., by adding the carrier material to the buffer solution. In that way, the carrier material will be formed simultaneously with and as part of the SP-D particles. Alternatively, the carriers may be separately prepared in a dry powder or liquid form and combined with the SP-D protein or nucleic acid by blending. The size of the carrier particles may be selected to improve the flowability of the powder or liquid, typically being in the range from 25 μ m to 100 μ m. Carrier particles in this size range will generally not penetrate into the alveolar region of the lung and will often separate from the SP-D protein or nucleic acid in the delivery device prior to inhalation. Thus, the particles which penetrate into the alveolar region of the lung will consist essentially of SP-D protein or nucleic acid and buffer. A preferred carrier material is crystalline mannitol having a size in the above-stated range.

The liquid or dry aerosol formulations of the present invention are preferably aerosolized by dispersion in a flowing air or other physiologically acceptable gas stream in a conventional manner. The liquid aerosol formulations will typically be used with a nebulizer. The nebulizer can be either compressed air driven or ultrasonic. Any nebulizer known in the art can be used in conjunction with the present invention such as but not limited to: Ultravent, Mallinckrodt, Inc. (St. Louis, Mo.); the Acorn II nebulizer (Marquest Medical Products, Engelwood Colo.). Other nebulizers useful in conjunction with the present invention are described in U.S. Pat. Nos. 4,624,251 issued Nov. 25, 1986; 3,703,173 issued Nov. 21, 1972; 3,561,444 issued Feb. 9, 1971 and 4,635,627 issued Jan. 13, 1971.

The formulation may include a carrier. The carrier is a macromolecule which is soluble in the circulatory system and which is physiologically acceptable where physiological acceptance means that those of skill in the art would accept injection of said carrier into a patient as part of a therapeutic regime. The carrier preferably is relatively stable in the circulatory system with an acceptable plasma half life for clearance. Such macromolecules include but are not limited to Soya lecithin, oleic acid and sorbitan trioleate, with sorbitan trioleate preferred.

The formulations of the present embodiment may also include other agents useful for protein stabilization or for the regulation of osmotic pressure. Examples of the agents include but are not limited to salts, such as sodium chloride, or potassium chloride, and carbohydrates, such as glucose, galactose or mannose, and the like.

10 Aerosol Dry Powder Formulations

It is also contemplated that the present pharmaceutical formulation will be used as a dry powder inhaler formulation comprising a finely divided powder form of the SP-D protein or nucleic acid and a dispersant. The form of the SP-D will generally be a lyophilized powder. Lyophilized forms of SP-D protein or nucleic acid can be obtained through standard techniques.

15 In another embodiment, the dry powder formulation will comprise a finely divided dry powder containing SP-D protein or nucleic acid, a dispersing agent and also a bulking agent. Bulking agents useful in conjunction with the present formulation include such agents as lactose, sorbitol, sucrose, or mannitol, in amounts that facilitate the dispersal of the powder from the device.

20 Pulmonary Therapy with SP-D protein or nucleic acid

The SP-D protein or nucleic acid of the invention is useful in the prophylactic or therapeutic treatment of SP-D mediated or SP-D related diseases or disorders in which pulmonary administration is desirable or in which the lungs are involved. The invention contemplates pulmonary administration of such amounts of the protein that are sufficient either to achieve systemic delivery of a therapeutic or biological amount of the protein, or such amounts that achieve only local delivery of a therapeutic or biological amount of the protein to the lung. The invention further contemplates parenteral administration or pulmonary administration of SP-D protein or nucleic acid for the treatment of emphysema. It will be appreciated by one skilled in the art that goal of systemic or local delivery will depend on the indication being treated.

30 What constitutes a therapeutically effective amount in a particular case will depend on a variety of factors within the knowledge of the skilled practitioner. Such factors include the physical condition of the subject being treated, the severity of the condition being treated, the disorder or disease being treated, and so forth. In general, any statistically significant attenuation of one or more symptoms associated with inappropriate SP-D activity or expression constitutes treatment within the scope of the present invention.

It is contemplated that SP-D protein or nucleic acid, or more preferably the formulations of the present invention, can be administered to a subject in need of prophylactic or therapeutic treatment. As used herein, the term "subject" refers to an animal, more preferably a mammal, and most preferably a human.

Pulmonary Administration of SP-D protein or nucleic acid can be used to result in systemic or local effects.

5 It is envisioned that the SP-D protein or nucleic acid will be delivered to achieve elevation of plasma levels of the protein to treat diseases or disorders that involve inappropriate SP-D activity or expression, i.e., extrapulmonary indications. In another embodiment of the present invention the SP-D is delivered via the airways to treat diseases or disorders involving SP-D when such diseases or disorders are manifest by local injury to the lung. As pointed out above, pulmonary administration of a SP-D is preferred for the treatment of lung disorders or diseases because of the
10 high local concentration of SP-D that can be delivered, the localization of significant amounts of the SP-D in extravascular space, and the ability to limit or minimize systemic effects of the SP-D.

Vectors for administration of SP-D nucleic acid

The present invention provides SP-D nucleic acid, which is delivered to a host cell via any of the above-mentioned aerosolization protocols. However, the nucleic acid may be delivered in a number of different forms.
15 Nucleic acids may be delivered as naked DNA or within vectors, the vectors including, but not limited to viral, plasmid, cosmid, liposome, and microparticles.

Isolation of Nucleic Acid Molecules of the Invention

20 A nucleic acid molecule encoding an SP-D polypeptide can be identified and isolated using standard methods, as described by Sambrook et al., (1989). For example, polymerase chain reaction can be employed to isolate and clone SP-D genes as described in U.S. Pat. No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite or complimentary strands of the template to be amplified. PCR can be used to amplify specific RNA
25 sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences and the like, to yield an amplification product. See also, Mullis et al., Cold Harbor Symp. Quant. Biol., 51:263 (1987); Erlich, ed., PCR Technology (Stockton Press, N.Y., 1989). Alternatively, the SP-D gene can be isolated from a library of the appropriate human or mammal, using an SP-D probe.

Nucleic acid molecules encoding amino acid sequence variants of an active SP-D polypeptide can be prepared
30 by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of an SP-D polypeptide.

To prepare expression cassettes and vectors for transformation herein, the recombinant or preselected
35 nucleic acid sequence or segment may be circular or linear, double-stranded or single-stranded. As used herein, a

"vector," "expression vector," or "expression cassette" is a replicon, or a genetic element that functions as an autonomous unit of DNA replication in vivo and capable of replication under its own control, such as a plasmid, a chromosome, a virus, phage or cosmid. Another DNA segment may be attached to the replicon or genetic element so as to bring about the replication of the attached segment.

5 Expression cassettes or expression vectors for host cells ordinarily include an origin of replication, a promoter located upstream from the SP-D coding sequence, together with a ribosome binding site, a polyadenylation site, and a transcriptional termination sequence. Those of ordinary skill will appreciate that some of the aforementioned sequences are not required for expression in certain hosts. For example, an expression vector for use with microbes need only contain an origin of replication recognized by the host, a promoter that will function in the
10 host and a selection gene.

 An expression cassette is constructed according to the present invention so that an human SP-D (or other appropriate SP-D) coding sequence is located in the cassette with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (e.g., RNA polymerase that binds to the DNA
15 molecule as the control sequences transcribes the coding sequence). As used herein, a DNA "coding sequence" is that portion of a DNA sequence, the transcript of which is translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino terminus) and a translation stop codon at the 3' (carboxy terminus). A coding sequence can include, but is not limited to, prokaryotic sequences, genomic DNA sequences from eukaryotic DNA, cDNA from eukaryotic
20 mRNA and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

 The control sequences can be ligated to the coding sequence prior to insertion into a cassette. As used herein, a coding sequence is "under the control" of the promoter sequence in a cell when RNA polymerase which binds the promoter sequence transcribes the coding sequence into mRNA which is then in turn translated into the protein
25 encoded by the coding sequence. Alternatively, the coding sequence can be cloned directly into an expression cassette that already contains the control sequences and an appropriate restriction site. Transformation may be by any known method for introducing polynucleotides into a host cell, including, for example, packaging the polynucleotide in a virus and transducing a host cell with the virus, and by direct uptake of the polynucleotide. The terms "transformed" or "transformation" or "stably transformed", as used herein, refer to the insertion of an exogenous polynucleotide into a
30 host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced into the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In prokaryotes and yeast, for example, the exogenous
35 DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably

transformed cell is one in which the exogenous DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosomal replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA. The transformation procedure used depends upon the host to be transformed. Bacterial transformation by direct uptake
5 generally employs treatment with calcium or rubidium chloride (Cohen Proc. Natl. Acad. Sci. USA, 69:2110 (1972)). Yeast transformation by direct uptake may be carried out using the method of Hinnen et al. Proc. Natl. Acad. Sci., 75:1929 (1978). Mammalian transformations by direct uptake may be conducted using the calcium phosphate precipitation method of Graham and Van der Eb Virology, 52:546 (1978), or the various known modifications thereof. Other methods for the introduction of recombinant polynucleotides into cells, particularly into mammalian cells, that
10 are known in the art include dextran-mediated transfection, calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the polynucleotides into nuclei.

As used herein, the term "recombinant nucleic acid," refers to a nucleic acid that has been derived or isolated from any appropriate source, that may be subsequently chemically altered in vitro, and later introduced into
15 target host cells. An example of recombinant DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment encoding SP-D, or a fragment or a variant thereof, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, for example, by the use of restriction endonucleases. The isolated sequence can then be further manipulated, such as, amplified for use in the invention, by
20 the methodology of genetic engineering.

As used herein, adenovirus is particularly suited as a vector for transforming the lung cells to express SP-D. Adenovirus naturally infects the cells of the respiratory tract and adenovirus can be aerosolized effectively to be administered to the lungs.

The preferred embodiments of the present invention will now be further described with reference to the
25 following examples. Example 1 describes the steps which resulted in the production of the SP-D (-/-) mouse.

EXAMPLE 1

SP-D (-/-) Knockout Mouse construction

SP-D (-/-) mice were generated by targeted gene inactivation. Integration of a pGKneo targeting vector
30 containing sequences from exon 2 of the SP-D gene generated a deletion of the second exon of the SP-D gene, which included removal of the initiating methionine and translation initiation sequences. The mouse SP-D gene sequence of Exons 1 and 2 can be found under Genbank accession No. AF047741. The targeting vector was designed using pGKneo by first subcloning a 5.1-kb blunt ended KpnI-tailed HindIII genomic fragment encoding intron 2 through exon 6 into a KpnI site between the neomycin-resistance cassette and the thymidine kinase cassette. Subsequently, a 1.5-kb
35 genomic PstI fragment containing a portion of intron 1 was tailed with XhoI linkers and cloned into an XhoI site 5' from

the neomycin-resistance cassette Eight of 104 ES clones surviving the double selection process were correctly targeted as determined by both 5' and 3' PCR analyses. Clone 93, a highly undifferentiated and proliferative clone, was expanded and injected into C57/Bl6 blastocysts generating chimeric males. Chimeric males were bred to NIH Swiss Black females. A female bearing the targeted gene was obtained and bred to NIH Swiss Black males to generate normal SP-D (-/-) and SP-D (\pm) mice. The distribution of genotypes from heterozygotic matings followed a Mendelian pattern, with 30 (+/+), 45 (+/-), and 25% (-/-) of 115 offspring, indicating that there were no obvious abnormalities in survival related to SP-D alleles.

SP-D(-/-) mice survive and breed normally in the vivarium under barrier containment facilities at Children's Hospital Medical Center, Cincinnati, Ohio. Mice have been viral free as assessed by serology. No serological evidence of viral infection in SP-D(-/-) mice was detected at necropsy.

To determine genotype, DNA from tail clips was digested with *Bam*HI and probed with a PCR product derived from genomic mouse DNA, containing exon 2 and part of intron 2, and with the G418 resistance cDNA clone. This demonstrated a simultaneous loss of exon 2 with appearance of sequences encoding G418 resistance in SP-D (\pm) and SP-D (-/-) mice.

To demonstrate that SP-D was not expressed in null animals, RNA blot analysis was conducted with total lung RNA from null, normal, and heterozygotic animals. The results showed approximately 50% reduction in the intensity of the SP-D hybridization band in heterozygous animals with a total absence of normally sized SP-D mRNA in null animals. After prolonged exposure, a diffuse mRNA band approximately 150 nucleotides smaller than the normal SP-D mRNA was detected. By scanning densitometry, this band represents less than 5% of the intensity of the normal SP-D transcript from heterozygous animals.

Western blot analysis of lung homogenates using rabbit anti-rat SP-D antiserum revealed SP-D was reduced approximately 50% in heterozygous SP-D (+/-) mice and was absent in SP-D (-/-) mice.

Both SP-D (-/-) and SP-D (+/-) mice survived normally in perinatal and postnatal periods. At selected ages, body, lung, and heart weights were obtained by direct measurement; and lung and heart volumes were obtained by fluid displacement. Lung protein and DNA content were assessed using bovine serum albumin and salmon sperm DNA, respectively, as standards. Body weights of SP-D (-/-) mice were slightly smaller prior to weaning, but were not significantly different from SP-D (+/+) mice after 3 weeks of age, Table 1. While lung volumes were not significantly different, lung-volume-to-body-weight ratios were increased in SP-D (-/-) mice at 3 and 6 weeks of age, Table 1. No significant differences were observed in heart volumes or heart-volume-to-body-weight ratios. At maturity (5 months), no changes in wet lung weight, total lung DNA or protein were noted.

Table 1: Comparison of Body Weights, Lung Volumes, and Volume-to-Body Weight Ratios (Mean \pm SE)

AGE	BODY WEIGHTS (g)		LUNG VOLUMES (ml)		LV:BW (ml/g $\times 10^{-3}$)	
	SP-D (-/-)	SP-D (+/+)	SP-D (-/-)	SP-D (+/+)	SP-D (-/-)	SP-D (+/+)
2 day	1.8 \pm 0.1*	3.4 \pm 0.1	ND	ND	ND	ND
5 day	3.7 \pm 0.3	4.6 \pm 0.2	ND	ND	ND	ND
7 day	3.9 \pm 0.2*	5.3 \pm 0.2	ND	ND	ND	ND
14 day	6.6 \pm 0.2*	7.7 \pm 0.2	ND	ND	ND	ND
17 day	10.9 \pm 0.5	10.6 \pm 0.7	0.36 \pm 0.02	0.36 \pm 0.03	3.25 \pm 0.05	3.36 \pm 0.03
3 wk	10.9 \pm 0.5*	14.1 \pm 1.2	0.36 \pm 0.01	0.37 \pm 0.03	3.43 \pm 0.21**	2.50 \pm 0.18
6 wk	23.2 \pm 0.6	24.7 \pm 0.5	0.63 \pm 0.03	0.58 \pm 0.02	2.71 \pm 0.13**	2.25 \pm 0.18
9 wk	25.2 \pm 1.2	27.8 \pm 1.3	0.55 \pm 0.03	0.61 \pm 0.02	2.10 \pm 0.16	2.20 \pm 0.09
28 wk	36.9 \pm 4.3	31.2 \pm 1.6	0.67 \pm 0.09	0.58 \pm 0.06	2.03 \pm 0.51	1.86 \pm 0.10

* Significant statistical differences were observed in body weights at 2 day, $p = 0.00001$; 7 day, $p = 0.0002$; 2 wk, $p = 0.007$; and 3 wk, $p = 0.04$. ** Significant statistical differences in LV:BW ratios were observed at 3 wk ($p = 0.02$), due to differences in body weight, and at 6 wk ($p = 0.03$), although body weights and lung volumes were not statistically different at this latter time point. $N = 3-7$ animals per group. LV:BW, lung volume-to-body weight ratio; ND, not determined.

However, while no abnormalities were observed in body weight, examples 2 through 5 describe the other abnormalities or changes found in SP-D (-/-) mice.

- 10 Example 2 demonstrates the effect on phospholipid levels. Alveolar and tissue phospholipid levels, specifically phosphatidylcholine pool levels, were markedly increased while total bronchoalveolar lavage (BAL) protein levels remained unchanged.

EXAMPLE 2

15 Phospholipid levels in the SP-D (-/-) Mouse

- Alveolar, tissue and total saturated phosphatidylcholine (Sat-PC) ($p < 0.001$) was increased about 3-fold in SP-D (-/-) mice. Levels of Sat-PC were not altered in SP-D (+/-) mice. For alveolar lavage phospholipid composition analysis, two to four samples consisting of the pooled lavage from two to three mice were evaluated for the relative abundance of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, and lyso-bis-phosphatidic acid. Phospholipid composition did not differ among genotypes. 20 Incorporation of (3 H)choline into total lung Sat-PC was slightly increased 8 hr following injection, incorporation being approximately 20% greater in SP-D (-/-) mice ($p < 0.05$).

- This result was completely unexpected in that previous work suggested a definite role for SP-A and a limited role for SP-D in lung phospholipid homeostasis. Previous diseases associated with surfactant homeostasis involved 25 accumulations of both surfactant proteins and lipids, thus the SP-D (-/-) null mouse demonstrates for the first time that

SP-D is an important player in surfactant lipid homeostasis and that surfactant lipid and protein homeostasis can be dissociated *in vivo*, since the total protein concentration in the surfactant did not change. However, there was a modest decrease in the total concentration of SP-A as explained in example 3.

5

EXAMPLE 3

Reduction in SP-A Levels in the SP-D (-/-) Mouse

No differences in SP-B and SP-C mRNAs or proteins were observed in SP-D (-/-) mice. In contrast, Northern blot hybridization of total lung RNA from SP-D (+/+), SP-D (+/-), and SP-D (-/-) mice and hybridization with and SP-A probe showed that SP-A mRNA was reduced in SP-D (-/-) mice. Consistent with the reduction in SP-A mRNA, BAL SP-A protein was apparently reduced by about 25% in SP-D (-/-) mice as assessed by Western blot analysis of alveolar lavage from three mice.

Therefore, SP-D has a role in the regulation of SP-A production. Since SP-A is involved in host defense in the lungs, SP-D can affect host defense in two ways. By up-regulation of SP-A production and by direct interaction with immune and microbial cells.

The ultrastructure of the phospholipid rich material isolated from the BAL of the SP-D (-/-) mice was evaluated as explained in example 4.

EXAMPLE 4

Changes in Surfactant Structure in SP-D (-/-) mouse

Large aggregate surfactant was isolated from pooled alveolar lavage of SP-D (-/-) and SP-D (+/+) mice and examined by EM using the technique outlined below. Lipid aggregates in SP-D (-/-) mice were enlarged and organized into electron dense phospholipid arrays and contained less tubular myelin compared with SP-D (+/+) mice. The ultrastructure proved to be markedly abnormal, containing reduced quantities of tubular myelin and forming unique densely packed lipid structures. Clearly, then, SP-D has a role in the structural organization of alveolar lipids.

25 Aggregate forms from alveolar lavage.

Surfactant in alveolar was can be separated into large aggregate (heavy, dense) and small aggregate (light, visicular) fractions by centrifugation. Alveolar washes were centrifuged at 40,000 x g over 0.8 M sucrose cushion for 15 min. The large aggregate surfactant then was collected from the interface, diluted with normal saline and centrifuged again at 40,000 x g for 15 min. The supernatant from the first 40,000 x g centrifugation that contains small aggregate surfactant is concentrated at 4°C by ultrafiltration using a 300,000 molecular weight retention filter (Minitan, Millipore Corp., Bedford, MA) or centrifugal concentrators (Amicon Corp., Danvers, MA). The small aggregate surfactant is diluted with 50 ml normal saline and ultrafiltered 3 times to remove soluble proteins.

Lastly, the structure of the lung was analyzed. Although, normal in SP-D (+/-) mice, increased numbers of large foamy alveolar macrophages and enlarged alveoli were observed in SP-D (-/-) mice. In example 5 the method and results for identifying lung abnormalities is outlined.

EXAMPLE 5

Lung Abnormalities in the SP-D (-/-) mouse

To determine whether absence of SP-D expression led to structural abnormalities, lungs from null, normal, and heterozygous mice were inflation fixed, and morphology and histochemical analysis was done on sections by light microscopy. There was no evidence of infection and no obvious alterations in airway epithelial cells at the level of light microscopy. However, heterogeneous abnormalities in lung parenchyma, with enlarged alveoli, were consistently observed in the SP-D (-/-) but not SP-D (+/-) or SP-D (+/+) controls.

Morphological and histochemical method

Lung tissue from SP-D (+/+) and SP-D (-/-) mice were sacrificed at 2 weeks, 3 weeks and 6 weeks. Animals were weighed, anesthetized with a 4:1:1 mixture of ketamine, acepromazine and xylazine, and exsanguinated by severing the inferior vena cava and descending aorta. The trachea was cannulated, and the lungs were collapsed by piercing the diaphragm. The lungs were inflation-fixed at 25 cm of water pressure with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 minute. The trachea was tied off as the cannula was removed in order to maintain the fixative in the inflated lung. Excised lungs and heart were allowed to equilibrate in cold fixative until they had sunk to the bottom of the container. Lung and heart volumes were then determined by fluid displacement. Each lobe was measured along its longest axis, bisected perpendicularly to the long axis, and processed into paraffin blocks. Five micron sections were cut in series throughout the length of each lobe, loaded onto polysine-coated slides, and stained with hematoxylin and eosin, Masson's trichrome stain for collagen, or ocein for elastin.

Lung morphology

In more detail, examination within the first 2 weeks of life demonstrated no detectable abnormalities in lung morphology, although increased numbers of normal appearing alveolar macrophages were noted in the alveoli of SP-D (-/-) mice at 14 days of age. In contrast abnormalities in lung histology were observed in SP-D (-/-) mice at 3 and 6 weeks of age consisting of enlarged airspaces and infiltration with atypical, foamy, alveolar macrophages. Enlarged airspaces associated with the accumulation of hypertrophic, foamy, alveolar macrophages and perivascular/peribronchiolar monocytic infiltrates were observed by 6 to 7 months of age, although the extent of airspace enlargement in individual SP-D (-/-) mice varied from moderate to severe in this age group.

In 7 month old SP-D (-/-) mice, subpleural fibrotic lesions were observed that stained intensely for collagen. Abnormalities in elastin deposition were also observed in the parenchyma of lungs from SP-D (-/-) mice at this time point. These consisted of regions of lung parenchyma with short thick, and more highly coiled elastic fibers, as well as regions of inflammation where elastin staining was decreased in adjacent alveolar septa (adjacent to macrophage accumulation and fibrosis).

Increased bronchial-associated lymphocytic tissue (BALT) was noted in the SP-D (-/-) mice. Intensity of SP-B immunostaining in type II cells was similar among the three genotypes. Type II cells were purified as outlined below. However, there were focal areas of increased numbers of large, foamy intra-alveolar cells, which appeared to be

alveolar macrophages containing abundant cytoplasmic vesicles. These cells increased in size as a result of increasing number and volume of cytoplasmic vesicles. The vesicles stained with Nile Red and fluoresced when excited with 520-550 nm green light after staining with Nile Blue and thus contained lipid or phospholipid. These macrophages were also stained by SP-B antiserum. In alveolar lavage, approximately 4-fold more macrophages (1.2×10^6 per mouse) were observed in SP-D (-/-) compared with normal mice (0.36×10^6 /mouse), but there were no changes in relative neutrophil or lymphocyte cell counts. Macrophage size was estimated from the diameter of fixed and stained macrophages from cytospin preparations sedimented onto glass slides at $1500 \times g$ for 2 min. Mean diameter of macrophages from (+/+) was $11.75 \pm 1.75 \mu\text{m}$ compared with (-/-) mice $18.75 \pm 7.25 \mu\text{m}$. Abnormally large macrophages, defined as those with a diameter of twice normal, comprised $22.4 \pm 0.6\%$ of the macrophages from (-/-) mice compared with $18 \pm 1.0\%$ from (+/+) mice. Numbers and morphology of alveolar macrophages were not different in SP-D (+/-) mice. Ultrastructural characteristics of type II cells were similar in SP-D (-/-) compared with SP-D (+/+) mice. The morphology of the alveolar macrophages is consistent with that of activated "foam" cells, known to be associated with inflammation.

Isolation of murine type II cells.

Type II cells are routinely isolated in this laboratory using the following method. Mice are anesthetized by intraperitoneal injection and pentobarbital (50 mg/ml 3.25 ml/kg body weight). After opening the abdominal cavity, mice are exsanguinated by severing the inferior vena cava. The trachea is exposed, cannulated with a 20 gauge luer stub adaptor, and secured by a suture. The chest plate is removed and lungs perfused with 10-20 ml sterile saline via the pulmonary artery until visually free of blood. Dispase (Collaborative Research, Inc., Bedford, MA) is instilled into the lungs via the tracheal catheter, followed by 1% low melt agarose, warmed to 45°C . Lungs are immediately covered with ice and incubated for 2 minutes to set the agarose. Lungs are dissected out, put in a culture tube containing an additional 1 ml Dispase, and incubated for 45 minutes at room temperature. Lungs are next transferred to a 60 mm culture dish containing 100 U/ml DNAase 1 (Sigma, St. Louis, MO) in 7 ml DMEM (Gibco BRL, Gaithersburgh, MD). The tissue is gently teased away from the airways and swirled for 5 minutes. Cells are then placed on ice until being filtered. The cell suspension is successively filtered through 100 μm and 40 μm cell strainers, and then through 25 μm nylon gauze (Tetko, Briarcliff Manor, NY). Cells are pelleted for 7 min at $130 \times g$ at 4°C and resuspended in 10 ml DMEM with 10% FBS (Intergen Co., Purchase, NY). Crude cell suspensions are added to 100 mm culture dishes that were previously coated with CD-45 and CD-32 antibodies (Pharmigen, San Diego, CA) and incubated for 102 hours at 37° in the presence of 5% CO_2 . Plates are removed from the incubator and gently "panned" to free settled type II cells. The cell suspension is centrifuged at $130 \times g$ at 4°C and resuspended in 10 ml DMEM with 10% FBS (Intergen Co., Purchase, NY). Crude cell suspensions are added to 100 mm culture dishes that were previously coated with CD-45 and CD-32 antibodies (Pharmigen, San Diego, CA) and incubated for 102 hours at 37°C in the presence of 5% CO_2 . Plates are removed from the incubator and gently "panned" to free settled type II cells. The cell suspension is centrifuged at $130 \times g$ for 7 minutes and cells are resuspended in DMEM containing 10% FBS.

Airspace and respiratory parenchyma

Morphometric measurements were performed on mice at 5 days (0.5 weeks), 14 days (2 weeks) and 17 days (2.5 weeks), 3 and 6 weeks, and 6 to 7 months of age. the overall proportion (% fractional area) of respiratory parenchyma and airspace was determined using a point counting method. Measurements were performed on sections
 5 taken at intervals throughout the left, right upper, or right lower lobes. Slides were viewed using a 20x objective, and the images (fields) were transferred by video camera to a computer screen using MetaMorph imaging software (Universal Imaging Corp., West Chester, PA). A computer-generated, 121-point lattice grid was superimposed on each field, and the number of intersections (points) falling over respiratory parenchyma (alveoli and alveolar ducts) or
 10 airspace was counted. Points falling over bronchioles, large vessels, and smaller arterioles and venules were excluded from the study. Fractional areas (% Fx Area) were calculated by dividing the number of points for each compartment (n) by the total number of points contained within the field (N), and then multiplying by 100:

$$\% \text{ Fx Area} = n/N \times 100$$

Ten fields per section were analyzed to gather the data. The x and y coordinates for each field measured were selected using a random number generator.

15 While, as shown in Figure 1, no differences in the relative proportion (% fractional area) of airspace (a) and respiratory parenchyma (b) were observed at 5 days (0.5 weeks), 14 days (2 weeks), or 17 days (2.5 weeks) of age, the % fractional area of airspace was increased significantly ($p=0.013$) in SP-D (-/-) mice by 3 weeks of age. More specifically, the fractional area devoted to both airspace (a) and parenchyma (b) diverged significantly between the two different genotypes at 3 weeks ($*p = 0.013$), 6 weeks ($*p = 0.0007$), and 28 weeks ($*p = 0.004$) of age.
 20 Likewise, the % fractional area of respiratory parenchyma was decreased in SP-D (-/-) mice compared to age-matched SP-D (+/+) controls (34% parenchyma/66% airspace compared to 42.5% parenchyma/57.5% airspace, respectively), Figure 1. Relative proportions of airspace and respiratory parenchyma continued to diverge significantly from controls at later time points, the % fractional areas ranging from 27% parenchyma/73% airspace to 37% parenchyma/63% airspace in 7 month old SP-D (-/-) mice ($n=5$). Age-matched SP-D (+/+) controls showed less variability, ranging from
 25 45% parenchyma/55% airspace to 47% parenchyma/53% airspace, at this time point ($n=4$). The overall percent reduction in parenchyma at 7 months of age in the SP-D (-/-) mice was 32% of control values, while the percent increase in airspace in the SP-D (-/-) mice was 27% of control values.

Cellular Proliferation

Animals were pre-injected with BrdU 4 hours prior to sacrifice in order to assess alterations in cellular
 30 proliferation. Immunohistochemical detection of incorporated BrdU was performed using a commercially available kit (Zymed Laboratories, Inc., San Francisco, CA). Sections of small intestine from each animal were immunostained in parallel with the lung sections as a positive control for BrdU incorporation.

BrdU labeling indices were relatively low, and no changes in BrdU labeling of respiratory parenchymal cells or alveolar macrophages were observed in the lungs from SP-D (-/-) mice compared to controls.

35

Lung volumes

Determination of lung volumes using pressure-volume curves was as follows: Twelve week-old mice were injected with sodium pentobarbital and placed in a chamber containing 100% oxygen to ensure complete collapse of alveoli by oxygen absorption. Mice were killed by exsanguination, the trachea cannulated and connected to a syringe
5 linked to a pressure sensor via a three-way connector (Mouse Pulmonary Testing System, TSS Incorporated, Cincinnati, OH). After opening the diaphragm, lungs were inflated in 75 μ l increments every 10 seconds to a maximum pressure of 28 cm of water and then deflated. Pressure-volume curves were generated for each animal, determining lung volumes (divided by body weight) at 10, 5, and 0 cm of water during the deflation curve. In figure 2, pressure-volume curves were generated in 5-6 mice at 12 weeks of age. Lung volumes associated with the deflation
10 limbs of pressure-volume curves were significantly greater for 12 week old SP-D (-/-) mice compared age-matched to SP-D (+/+) mice at 10 cm H₂O and at the maximum pressure of 28 cm H₂O (*p < 0.05).

Statistically significant differences were determined by using either analysis of variance for fractional areas and pressure-volume curves, followed by the Student-Newman-Keuls procedure, or the student's T test for comparison of body weights, lung and heart volumes, volume:body weight ratios, total protein and DNA content. Differences of
15 p < 0.05 were considered significant. Values are given as mean \pm SE.

Increased lung volumes were readily apparent in SP-D (-/-) mice at 12 weeks of age, consistent with histologic and morphometric studies demonstrating emphysema, see Figure 2.

Alveoli

The enlarged alveoli were consistently observed in the SP-D (-/-) mice. This provides evidence for the
20 involvement of SP-D in the regulation of alveolar remodeling in the lungs. Because abnormalities and airspace remodeling is a defining characteristic of emphysema, the SP-D (-/-) mouse is an ideal model for emphysema.

EXAMPLE 6

Cytokines, Hydrogen Peroxide Production, and Metalloproteinase Activities:

25 Cytokine measurements

Lung homogenates from 6 to 9 week-old mice were centrifuged at 2000 RPM and stored at -20°C. Tumor necrosis factor alpha (TNF- α), interleukin (IL)-1 β , IL-6, and macrophage inflammatory protein (MIP)-2 were quantitated using murine sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. All
30 plates were read on a microplate reader (Molecular Devices, Menlo Park, CA) and analyzed with the use of a computer-assisted analysis program (Softmax; Molecular Devices). Only assays having standard curves with a calculated regression line value of > 0.95 were accepted for analysis.

Hydrogen Peroxide Production

Alveolar macrophages were collected by bronchoalveolar lavage with 1 ml of dye-free RPMI media (Gibco, Grand Island, NY) times three. Bronchoalveolar lavage fluid (BALF) from 8-10 mice was pooled to provide sufficient
35 numbers of macrophages for analysis. The lavage was centrifuged at 1200 RPM for 10 minutes and one million

macrophages were resuspended in PBS. Hydrogen peroxide production by macrophages was measured using a commercially available assay (Bioxystech H_2O_2 -560 assay, OXIS International, Portland, OR), based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by hydrogen peroxide under acidic conditions. Methods followed the manufacturer's recommendations. Hydrogen peroxide production was determined after activation with 100 ng/ml phorbol myristate acetate (PMA) or without stimulation.

5 Metalloproteinase Activity

Mouse lavage samples were centrifuged (100,000 x g, 1 hour) in a SW-28 rotor (Beckman, Palo Alto, CA). The supernatants were concentrated using Centricon-30 filtration units (Amicon, Inc., Beverly, MA). Samples (200 μ g protein) were electrophoresed under nonreducing conditions (laemmli) into 10% Zymogram, gelatin and casein gels (Novex, San Diego, CA). Following electrophoresis, gels were washed twice with 2.5% Triton X-100 (37°C, 15 min.) and incubated for 16 hours with 40 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1mM $ZnCl_2$. Gels were stained with 0.5% (w/v) Coomassie Blue in 50% methanol, 10% acetic acid for 1 hour, then destained. Metalloproteinases were detected as clear bands against the blue background. Metalloproteinase 2 and 9 mRNA's were quantitated by Northern blot analyses of total lung mRNA from wild type and SP-D (-/-) mice using [^{32}P]-labeled cDNA probes (Chemicon International, Inc., Temecula, CA).

15 Results

At 6 to 9 weeks of age, lung homogenates from SP-D (-/-) mice did not contain inflammatory levels of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 or MIP-2, although basal levels of IL-1 β were increased significantly, Figure 3. In contrast, oxidant production, as assessed by measuring hydrogen peroxide production by alveolar macrophages isolated from SP-D (-/-) mice, was increased 10 fold, Figure 4. Hydrogen peroxide and superoxide production is a measure of macrophage activation, particularly the microbicidal activation. Since oxidant production has been associated with activation of a number of metalloproteinases and with emphysema in both human and animal studies, metalloproteinase activities were estimated by degradation of gelatin substrates after SDS-PAGE of BALF supernatants isolated from SP-D (-/-) and SP-D (+/+) mice. Bands of activity consistent with metalloproteinases -2 and -9 were readily detected in both genotypes, but were not altered in BALF from SP-D (-/-) mice. Likewise, the abundance of metalloproteinases -2 and -9 mRNA's were similar in whole lung RNA samples from SP-D (-/-) and SP-D (+/+) mice as assessed by Northern blot analysis. However, production of MMP-2 and 9 by alveolar macrophages isolated from SP-D (-/-) mice were markedly increased. Likewise, immuno-staining for MME (macrophage metallo-elastase) and MMP-9 were increased in the lungs of the SP-D (-/-) mice.

The results in Examples 1-6 were completely unexpected. There is nothing in the literature to suggest an SP-D null mouse is a model for emphysema.

In summary, the SP-D (-/-) mouse conclusively demonstrates a remarkable and surprising role for SP-D in regulation of surfactant homeostasis, the structure of alveolar surfactant in the lung, regulation of SP-A expression, or plays a critical inhibitory role in oxidant, hydrogen peroxide production in the lung. SP-D levels are important for suppression of ongoing oxidant production and injury and the regulation of alveolar remodeling and suppression of

proteases that cause emphysema. This makes the SP-D (-/-) mouse an excellent model for emphysema. Example 7 will summarize the results for the mouse model of emphysema.

EXAMPLE 7

5 SP-D (-/-) mouse as a model for Emphysema

SP-D deficiency caused inflammation, increased oxidant production by isolated alveolar macrophages, emphysema, and localized fibrosis in gene-inactivated SP-D (-/-) mice. The timing and progressive nature of these pulmonary abnormalities support the conclusion that alveolar enlargement in SP-D (-/-) mice is caused by alveolar remodeling associated with chronic inflammation, rather than with development abnormalities occurring during
10 alveologenesis. The present findings are consistent with an important and unanticipated role of SP-D in the modulation of pulmonary inflammation and oxidant production and suggest that changes in the regulation or function SP-D may play a role in the pathologic processes leading emphysema following chronic lung injury.

Histologic and morphometric analyses of lungs from SP-D (-/-) mice revealed no abnormalities in lung structure until 3 weeks of postnatal age, one week after alveologenesis is completed in the mouse. This was
15 consistent with the observation that the relative proportions of respiratory parenchyma and airspace were similar in both SP-D (-/-) and SP-D (+/+) mice between postnatal days 5 and 17. After 2 weeks of age, increased parenchymal-airspace ratios were observed in SP-D (-/-) mice, consistent with ongoing remodeling of the parenchyma and alveolar spaces. Enlarged airspaces were generally associated with focal accumulation of large, foamy, alveolar macrophages, although there was some heterogeneity in both localization and extent of inflammatory infiltrates and remodeling in
20 older mice. While focal accumulation of alveolar macrophages in lungs of SP-D (-/-) mice were observed as early as 2 weeks of age, macrophage morphology remained normal at this time. Abnormal alveolar macrophage morphology, consisting of enlarged foamy cells, was noted by 3 weeks of age and was coincident with enlargement of alveolar structures thereafter. Previous studies demonstrated increased numbers of enlarged alveolar macrophages in SP-D (-/-) mice by 8 weeks of age. Thus, the development of emphysema in SP-D (-/-) mice is consistent with the temporal and
25 spatial accumulation of activated macrophages, and increased production of proteases MMP-2, 9 and MME, suggesting their role in the remodeling process. The present findings do not support a role for SP-D in normal lung morphogenesis and alveologenesis, a process generally completed by approximately 2 weeks of postnatal age in mice.

The present findings do support an important role for SP-D in the modulation of alveolar macrophage activation and oxidant production, leading to protease activation, emphysema and fibrosis. Macrophage infiltration
30 and lung remodeling in SP-D (-/-) mice were associated with modest but significant differences in inflammatory levels of various pro-inflammatory mediators, including IL-1b, MIP-2, but not TNF- α and IL-6, but rather with markedly increased hydrogen peroxide production by isolated alveolar macrophages. Although basal levels of IL- β 1 were significantly increased in SP-D (-/-) mice, IL- β 1 was not increased to levels typically detected in severe inflammation. While increased IL-1 β and hydrogen peroxide production were observed in SP-D (-/-) mice, it remains unclear whether
35 the pulmonary abnormalities seen in these mice were directly mediated by cytokine or oxidant-induced injury.

Although SP-D has been proposed to play an important role in host defense, there was no histologic or serologic evidence of infection in the SP-D (-/-) colony.

Enhanced hydrogen peroxide production and increased numbers of alveolar macrophages found in the lungs of SP-D (-/-) mice support the concept that SP-D plays a critical anti-inflammatory role in the lung and regulates hydrogen peroxide production by alveolar macrophages *in vivo*. Relationships between oxidant injury and the development of emphysema and pulmonary fibrosis are well established in numerous animal and genetic models. For example, neonatal exposure to hyperoxia caused alveolar remodeling and fibrosis in newborn mice. Since activation of metalloproteinases has been associated with oxidant injury and emphysema, metalloproteinase activities were assessed in BALF from the SP-D (-/-) mice. While protease activity consistent with metalloproteinase -2 and -9 were readily detected by zymography in lung homogenates, but, no consistent changes in the activities of these proteinases or their mRNAs were detected in SP-D (-/-) mice. However, markedly increased production of MMP-2 and MMP-9 were demonstrated in isolated alveolar macrophages from SP-D(-/-) mice *in vitro* and immuno-staining for MME and MMP-2 were increased in the lungs of SP-D(-/-) mice *in vivo*. Thus, localized increased concentrations of metalloproteinases and/or alterations in other proteases or antiproteases is associated with SP-D deficiency. Deficiencies in antiproteases, as well as smoking and oxidant injury from oxidizing toxicants (e.g., bleomycin or paraquat), have all been associated with emphysema or pulmonary fibrosis in human lung.

While surfactant phospholipid content was increased in SP-D (-/-) mice and was associated with increased numbers of large, foamy, alveolar macrophages, increased phospholipid content alone is not likely to be sufficient to cause the alveolar remodeling observed in SP-D (-/-) mice. In fact, the overall effect of surfactant phospholipids appears to be anti-inflammatory, altering phagocytosis, oxidant production, and cytokine release, and inhibiting lymphocyte proliferation, immunoglobulin production, and expression of adhesion molecules. On the other hand, transgenic mice in which GM-CSF was over-expressed in the respiratory epithelium had markedly increased numbers of normal appearing alveolar macrophages, but did not develop pulmonary alveolar proteinosis/lipoidosis, emphysema, or fibrosis. In contrast, surfactant phospholipids and proteins were markedly increased in lungs from both GM-CSF (-/-) and GM-receptor common beta subunit (βc) deficient mice in association with alveolar macrophage accumulation and perivascular/peribronchiolar monocyte infiltrates; however, neither model of pulmonary alveolar proteinosis/lipoidosis was associated with emphysema or fibrosis. Likewise, transgenic mice over-expressing IL-4 in the lung also exhibited increased amounts of surfactant protein and lipids, as well as increased numbers of inflammatory cells, but did not develop emphysema.

Although concentrations of SP-D in the lung change during development, increasing with advancing age, SP-D levels are also influenced by various clinical conditions. Recent studies demonstrated marked reduction of SP-D concentrations in BALF obtained from patients with cystic fibrosis (CF), supporting a potential role for SP-D in the pathogenesis of the chronic inflammation associated with CF lung disease. SP-D levels were also reduced in BALF of smokers, suggesting that decreased levels of SP-D may contribute to later development of chronic obstructive pulmonary disease (COPD) and emphysema in these patients. Although concentrations of SP-D in BALF were

increased in patients with pulmonary alveolar proteinosis (PAP), patients with idiopathic pulmonary fibrosis (IPF) and interstitial pneumonia associated with collagen vascular disease (IPCD) had decreased BALF levels of SP-D. On the other hand, serum concentrations of SP-D were increased in patients with PAP, IPF, and IPCD; although serum levels of both SP-A and SP-D varied with the severity of IPF and during the course of anti-inflammatory therapies. These clinical findings, as well as the present study, demonstrating that SP-D is required for maintenance of normal lung architecture and suppression of oxidant production, support the concept that changes in SP-D concentrations may be involved in the pathogenesis of lung injury associated with various clinical conditions, including oxidant injury, lung abscesses, secondary diseases, cystic fibrosis, interstitial pulmonary fibrosis (IPF), and chronic obstructive pulmonary disease (COPD), various lung infections, respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), chemotherapy-induced lung injury, lung fibrosis secondary to primary abscess (ie: sarcoid), and asthma.

In our previous studies, no abnormalities in alveolar macrophages or lung morphology were observed in the heterozygous SP-D (+/-) mice, demonstrating that a 50% reduction in SP-D concentration in BALF is not sufficient to cause pulmonary abnormalities. The precise concentrations of SP-D that are required for inhibition of oxidant-induced injury and lung remodeling are unclear at present. Whether further injury or oxidant stress to the lungs of SP-D (+/-) or SP-D (-/-) mice will exacerbate emphysema and fibrosis in this animal model remains to be determined.

The modest reduction of lung SP-A concentrations found in SP-D (-/-) mice is not likely to contribute to the changes in lung morphology observed in these mice, since neither SP-A (+/-) nor SP-A (-/-) mice developed emphysema. Furthermore, lung morphology of SP-A deficient mice was normal, and, in contrast to SP-D (-/-) mice, SP-A deficiency was associated with decreased hydrogen peroxide production by isolated alveolar macrophages.

SP-D (-/-) mice developed severe and progressive emphysema. Alveolar remodeling and macrophage abnormalities were apparent as early as 3 weeks of age, while mild, focal, pulmonary fibrosis was observed at 6 to 7 months of age, demonstrating a role for SP-D in the regulation of inflammation and alveolar remodeling. The present study also demonstrated an unexpected role for SP-D in the regulation of hydrogen peroxide production by alveolar macrophages *in vivo*, which may contribute to the development of emphysema in the lungs of SP-D (-/-) mice. Whether SP-D deficiency contributes to ongoing inflammation or to the development of emphysema and fibrosis found in various human chronic lung diseases, including those caused by smoking and other oxidants, remains to be determined.

Because of the lack of pharmaceutical therapies for the treatment of emphysema, a model for testing possible therapies is imperative. The SP-D (-/-) mouse provides that model. Example 8 provides a sample framework for testing pharmaceuticals, protein preparations, or genetic manipulations for the treatment of emphysema.

EXAMPLE 8

Testing Therapies in the Mouse Model

A number of doses or concentrations of protein or pharmaceutical diluted in an appropriate buffer is administered to SP-D (-/-) mice intratracheally. Protein and pharmaceutical is purified as appropriate for *in vivo* use. Recombinant adenovirus or other genetic vectors containing the gene of interest is administered as follows. SP-D (-/-)

mice are immunosuppressed to block specifically T cell-mediated immune responses, and treated with an adenoviral construct designed to express the gene of interest in transduced cells. Mice are injected intraperitoneally with H57 antibody 3 days prior to receiving the adenoviral construct. H57 alters immune recognition at the T cell receptor and decreases splenic and lung T and B lymphocytes. One dose is instilled intratracheally and another group is treated intraperitoneally with H57 followed by intratracheal administration of vehicle alone. Levels of the protein of interest is measured 1 week after administration to detect uptake and expression of the vector. Four mice are tested and untreated SP-D (-/-) mice are used as a control. Intratracheal inoculation involves anesthetizing with isoflurane, and an anterior midline incision is made to expose the trachea. A 30-gauge needle attached to a tuberculin syringe is inserted into the trachea, and a 100- μ l inoculum of protein or pharmaceutical is dispersed into the lungs. The incision is closed with one drop of Nexaband. Nonpyogenic PBS is injected intratracheally as a control.

To test for efficacy of the protein, pharmaceutical, or genetic manipulation at diminishing the effects of emphysema, a number of tests are performed.

To determine the effects of the protein or pharmaceutical on the lung structure lungs are inflation fixed and sections evaluated by electron microscopy. Lungs from treated and untreated mice are inflated via a tracheal cannula at 20 cm of pressure with 4% paraformaldehyde and removed en bloc from the thorax. Lungs are dehydrated and embedded in paraffin. Tissue sections (5 μ m) are stained with hematoxylin and eosin.

To test the number and morphology of macrophages: Staining with Nile Red detects vesicles and staining with Nile Blue and exciting with 520-550 nm green light is an additional method to detect lipid or phospholipid. Macrophage number is determined by staining with anti-MAC-1 or other macrophage-specific antiserum. Macrophage size is estimated from the diameter of fixed and stained macrophages from cytopsin preparations sedimented onto glass slides at 1500 x g for 2 min.

Surfactant composition and ultrastructure is analyzed as follows: The structure of surfactant is analyzed by isolating large aggregates from pooled alveolar lavage of SP-D (-/-) treated and untreated mice and examined by EM (see protocol below). For alveolar lavage phospholipid composition analysis, two to four samples consisting of the pooled lavage from two to three mice are evaluated for the relative abundance of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, and lyso-bis-phosphatidic acid. Incorporation of (³H)choline into total lung Sat-PC is evaluated to determine total phospholipid concentration. Aggregate forms from alveolar lavage.

Surfactant in alveolar wash can be separated into large aggregate (heavy, dense) and small aggregate (light, visicular) fractions by centrifugation. Alveolar washes were centrifuged at 40,000 x g over 0.8 M sucrose cushion for 15 min. The large aggregate surfactant then was collected from the interface, diluted with normal saline and centrifuged again at 40,000 x g for 15 min. The supernatant from the first 40,000 x g centrifugation that contains small aggregate surfactant is concentrated at 4°C by ultrafiltration using a 300,000 molecular weight retention filter (Minitan, Milfore Corp., Bedford, MA) or centrifugal concentrators (Amicon Corp., Danvers, MA). The small aggregate surfactant is diluted with 50 ml normal saline and ultrafiltered 3 times to remove soluble proteins.

SP-D as a Treatment for Pulmonary Diseases

Because deletion of SP-D produced the mouse model for emphysema, SP-D is an obvious choice as a treatment for or prevention of emphysema. It is also an obvious treatment for other types of pulmonary disease since many of these diseases are characterized by aberrant surfactant production. In addition, its affect on SP-A and its possible role in host defense makes it a useful tool to augment immune function in the lungs. The feasibility of gene transfer to the respiratory epithelium is very promising as a treatment for various pulmonary diseases. A variety of viral and non-viral-based vectors have been developed to transfer genes to cells of the airways, including recombinant adenoviral vectors. These vectors are particularly promising for use in respiratory treatment because they have the potential of being aerosolized. Example 9 is an experiment using purified mouse SP-D protein for treatment of emphysema in SP-D(-/-) mice. Example 10 is an experiment using adenovirus to express rat SP-D for treatment of emphysema in SP-D(-/-) mice. Example 11 provides a sample framework for the use of SP-D peptide, or vectors expressing SP-D for the prevention and treatment of these diseases. Emphysema is used as an exemplary pulmonary disease. Adenovirus is used as an exemplary vector.

EXAMPLE 9

Treatment with purified SP-D

SP-D(-/-) mice were treated with purified mouse SP-D, purified as outlined below. Saturated PC levels were analyzed in alveolar lavage and total lung lavage. Repeated doses intratracheally at 24 hour intervals resulted in partial correction of lipid accumulation after 3 to 7 doses, see Figure 12.

The half life of SP-D in the airway was determined as 13 hours in mouse (see Figure 13) (the technique is outlined below); therefore, the SP-D deficiency can be treated by replacement of SP-D protein at a reasonable interval by aerosol or particulate inhaler or surfactant mixtures.

Purification of mouse SP-D

Mouse bronchoalveolar lavage (BAL) fluid from GMCSF and SP-A double null mutant mice was collected, frozen, and pooled for later purification of SP-D. Maltosyl-agarose (Sigma) was packed in a gravity flow column (10 x 80 mm) and equilibrated with buffer containing 20 mM Tris-HCl, pH 7.4, 10 mM calcium chloride, 0.02% (W/V) sodium azide (TCB). The BAL was made 20 mM with respect to Tris-HCl, and 10 mM with respect to EDTA, pH 7.4 and stirred for one hour at room temperature. The turbid solution was centrifuged at 10,000 X g for 40 minutes at 4 degrees C. The supernatant was made 20 mM with respect to calcium chloride and readjusted to pH 7.4 before loading on the maltosyl-agarose column. The column was washed to background absorbance with TCB followed by washing with TCB containing 1.0 M Sodium Chloride. The SP-D, which has a specific requirement for calcium in binding to maltose was eluted with 50 mM manganese chloride, 20 mM Tris-HCl, 0.02% (W/V) sodium azide, pH 7.4. The fractions containing SP-D were determined by SDS polyacrylamide gel electrophoresis or by direct ELISA, pooled, and dialysed against three changes of 20 mM Tris-HCl, 100 mM sodium Chloride, 5 mM EDTA pH 7.4. This protocol

was adapted from Strong, Peter; Kishore, Uday; Morgan, Cliff; Bernal, Andres Lopez; Singh, Mamta; and Reid, Kenneth B.M.; Journal of Immunological Methods 220 (1998) 139-149.

Treatment of mice with surfactant components.

We have successfully used a technique for oral blind intubation using 26 g feeding tubes in mice under anesthesia with isoflurane for repetitively treating mice with SP-D daily for up to 7 days without problems. This approach avoids surgery and permits the type of experiments proposed for SP-D replacement and treatment with mutant SP-D proteins.

Initially SP-D(-/-) mice were treated with purified mouse SP-D by tracheal instillation. Three or more doses of 2.9 g SP-D given at 24 hour intervals decreased both alveolar and saturated PC pools (see Figure 14). This dose of SP-D given is approximately the amount present in the endogenous pool in SP-D(+/+) mice. Given the lung association and clearance kinetics, this is a low dose. Thus exogenous administration of SP-D directly influences surfactant lipid metabolism and provides an experimental model in which we can test the function of modified SP-D molecules *in vivo*.

Biological half-life protocol:

We have measured the biological half-life of SP-D in mice in order to design experiments for treatment with SP-D. We iodinated purified mouse SP-D with ¹²⁵I using the Bolton-Hunter reagent as we have done previously for SP-A and the other surfactant proteins. The clearance of SP-D from alveolar lavages of SP-D(+/+) and SP-D(-/-) mice was similar with a half life of about 13 hours (see Figure 13). The $t^{1/2}$ of 17 h for SP-D in the lungs of SP-D(-/-) mice was somewhat longer than the $t^{1/2}$ of 13 hours for SP-D(+/+) mice.

GM-CSF deficiency causes a 48 fold increase in SP-D, and the GM-CSF(-/-) x SP-A(-/-) cross has similarly elevated SP-D but no SP-A. We have isolated SP-D from alveolar washes from GM-CSF(-/-) x SP-A(-/-) mice in high purity and in large amounts by the methods described by Persson et al. using an affinity column of mannose-Sepharose 6B in the presence of Ca²⁺.

EXAMPLE 10

Treatment with SP-D expressed from an adenovirus

A new adenovirus expressing rat SP-D was constructed. The virus produced SP-D in cells and in the lungs of normal or SP-D deficient mice. Western blots showed that the rat SP-D was produced in 293 cells as well as in mice.

Construction of Ad-rSPD adenovirus (see Figure 14)

Wild type rat SPD cDNA was liberated from plasmid WT-rSPD/pG32 with EcoR I digestion and the 3' ends filled in with Klenow. The 1.3 kB rSPD cDNA was inserted into the EcoR V site of plasmid pAvS6a to make plasmid pAvS6a-rSPD. Plasmid pAvS6a-rSPD has a RSV promoter, a rSPC cDNA, an SV40 poly A signal and an Ad5 sequence (9.24-17.34 mu). Not I linearized pAvS6a-rSPD was co-transfected into 293 cells with Cla I digested large fragment of adenoviral DNA Ad dl327, which has E3 region (78.5-84.7 mu) deleted. After homologous recombination, individual plaques were analyzed by Western blot assay to determine rSPD protein expression. One rSPD positive clone was subject to one round of plaque purification. The Ad-rSPD adenovirus has deletions in E1 and E3 regions and

is replication deficient. After amplification in 293 cells, the purified Ad-rSPD adenovirus was produced through two rounds of CsCl gradient ultracentrifugation. The adenovirus expressed SP-D and therefore could be used to restore pulmonary abnormalities by intratracheal administration. Therefore, this remains a very positive possibility for treatment of emphysema and many other SP-D deficiency illnesses as well as various other forms of pulmonary injury and deficiency.

EXAMPLE 11

Treatment with SP-D expressed from other vectors, proteins, or pharmaceuticals

The temporal, spatial and stoichiometric requirements for SP-D in the restoration of phospholipid homeostasis were determined in example 9. Initial studies to determine the kinetics of clearance of SP-D were performed with ¹²⁵I labeled SP-D administered intratracheally; half-life was calculated and the information used in design of SP-D replacement experiments. The dose of SP-D required to achieve normal physiologic concentrations of SP-D after administration was clarified.

Administration of purified SP-D protein was used to treat various pulmonary disease in Example 9. However, physiologic abnormalities in pulmonary disease may require long term correction of SP-D in the lungs. Therefore, recombinant adenovirus or other genetic vectors containing the mammalian SP-D gene will be used (see Example 10 and 11). Recombinant adenovirus vectors used Clara cell secretory protein (CCSP) and SP-C promoters to selectively express SP-D in bronchiolar (Clara cell) and alveolar (Type II cell) compartments (see Example 10). Three days prior to treatment with adenoviral vector the mice are immunosuppressed by injection intraperitoneally with 200 ug of monoclonal anti-T cell receptor antibody, H57. Adenovirus was administered by intratracheal injection of 5×10^6 PFU of virus. Levels of SP-D protein were measured 1 week after administration to detect uptake and expression of the vector. Four mice were tested and SP-D (-/-) mice receiving no treatment are used as a control. To test for efficacy of the SP-D at diminishing the effects of emphysema, a number of tests are performed as follows.

To determine the effects of a protein or pharmaceutical on the lung structure (Example 11), lungs are inflation fixed and sections evaluated by electron microscopy. Lungs are inflated via a tracheal cannula at 20 cm of pressure with 4% paraformaldehyde and removed en bloc from the thorax. Lungs are dehydrated and embedded in paraffin. Tissue sections (5µm) are stained with hematoxylin and eosin.

Number and morphology of macrophages are analyzed. Staining with Nile Red detects vesicles and staining with Nile Blue and exciting with 520-550 µm green light is an additional method to detect lipid or phospholipid. Macrophage number is determined by direct counting or macrophage cell surface markers. Macrophage size is estimated from the diameter of fixed and stained macrophages from cytopsin preparations sedimented onto glass slides at 1500 x g for 2 min.

Surfactant composition and ultrastructure are analyzed as follows: the structure of surfactant is analyzed by isolating large aggregates from pooled alveolar lavage of SP-D (-/-) treated and untreated mice and examined by EM. For alveolar lavage phospholipid composition analysis, two to four samples consisting of the pooled lavage from two to

three mice are evaluated for the relative abundance of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, and lyso-bis-phosphatidic acid. Incorporation of (³H)choline into total lung Sat-PC is evaluated to determine total phospholipid concentration.

Once efficacy of the treatment is determined, treatment can be tested on other appropriate mammals.

5 Involvement of SP-D in Pulmonary Infection

The role of SP-D and SP-A in host defense in the lungs has been repeatedly demonstrated. SP-A and SP-D have specific interactions with various microorganisms *in vitro*, modifying pulmonary inflammation *in vitro* by altering cytokine and free radical production. The role of SP-D in bacterial clearance and inflammatory response of the lung was evaluated *in vivo* using a mouse model of SP-D deficiency. SP-A-deficient mice are known to be more susceptible to infections. A number of *in vitro* studies have shown a possible role for SP-D in host defense in addition to its role in up-regulating SP-A. Examples 8-11 outline sample protocols for testing SP-D as a therapy in the, bacterially, or fungally infected SP-D (-/-) mice as well as in the SP-A (-/-) mice. Examples 12-14 are experiments showing the role of SP-D in the response to bacterial, fungal, and viral infection. Example 13 is an experiment showing the effect of infecting SP-D(-/-) mice with Respiratory Syncytial Virus.

15

EXAMPLE 12

Clearance of bacterial agents from SP-D(-/-) mice

SP-D deficient mice (SP-D -/-) were intratracheally infected with Group B streptococcus (GBS) or *Hemophilus influenzae* (Hfln) to assess clearance compared to wild type mice. Group A Streptococcus was administered at 10⁴ CFU. Pulmonary inflammation was also assessed by analysis of BAL fluid for total cells (Figures 5, 6, and 7), cytokine levels in lung homogenates (Figure 8), oxygen radical production by alveolar macrophages (Figure 11) and Nitrite levels in BAL (Figure 9).

SP-D -/- mice cleared the bacteria similarly to wild type mice (see Figures 5 and 6). Infection with GBS and Hfln resulted in significantly greater total cells in the BAL fluid of the SP-D -/- mice compared to wild type mice (figure 7). Selective alterations of cytokine levels were detected in SP-D -/- mice. Tumor necrosis factor (TNF-) and interleukin (IL)-6 levels were greater in lung homogenates from SP-D -/- mice early after infection with GBS or Hfln (Figure 8). Macrophage inflammatory protein-2 (MIP-2), a neutrophil chemoattractant, was significantly greater in lung homogenates from SP-A -/- mice after Hfln but not GBS infection (Figure 8). Macrophages from SP-D -/- mice generated significantly greater superoxide and hydrogen peroxide compared to wild type mice (Figure 11).

BAL nitrite levels were increase in SP-D (-/-) mice as compared to wildtype mice. Nitric oxide production was measured as nitrite in BALF. Nitric oxide plays a role in host defense by contributing to bacterial killing. Nitric oxide reacts with superoxide to form peroxynitrite which is a potent bacteriocidal agent.

In figure 10 phagocytosis was evaluated using light microscopy and flow cytometry. SP-D(-/-) mice showed significantly reduced phagocytosis of bacteria as compared to wildtype.

Therefore, in the absence of SP-D increased inflammatory responses were observed following bacterial infection of the lung with GBS or Hflu. Production of reactive oxygen species by alveolar macrophages was enhanced in SP-D ^{-/-} mice. These results support a critical and distinct role of SP-D in pulmonary immune and inflammatory responses to bacterial infection, *in vivo*.

5 In Example 13, the SP-D^{-/-} mice were infected with Respiratory Syncytial Virus.

Host defense mechanisms have evolved to maintain the lung clear of microbial pathogens including innate mediators of bacterial and viral clearance and acquired immune responses.

EXAMPLE 13

10 Clearance of virus from SP-D^{-/-} mice

SP-D^{-/-} mice were intratracheally infected with respiratory syncytial virus (RSV), a common respiratory pathogen in children. Viral titers and lung inflammation were assessed in SP-D ^{-/-} mice and wild type mice. RSV titers in lung homogenates were significantly increased in SP-D ^{-/-} compared to wild type mice 3 and 5 days after administration. However, significantly increased numbers of inflammatory cells were found in BAL fluid from SP-D ^{-/-} mice with a greater percentage of PMNs compared to wild type mice, 3 and 5 days after RSV infection. In addition, lung inflammation assessed by histology, 5 days after RSV infection was greater in SP-D ^{-/-} compared to wild type mice. Pro-inflammatory cytokines, including TNF- α , IL-1, IL-6 and MIP-2 were greater in lung homogenates from SP-D ^{-/-} mice 3 and 5 days after RSV infection. SP-D ^{-/-} mice had less efficient viral clearance from the lung and demonstrated greater inflammatory responses following RSV infection than wild type mice. These findings demonstrate that SP-D plays an important role in innate defense and regulation of inflammation in the lung after RSV infection *in vivo*. Similar findings were observed after Influenza and adenovirus infected the lung.

EXAMPLE 14

Clearance of Fungi from the SP-D^{-/-} mice

25 The mouse is infected as follows: an appropriate prototype of a fungal pathogen is used. The infectious agent is purified as appropriate and suspended in appropriate buffer and administered intratracheally with or without SP-D into the SP-D ^{-/-} mouse (as in Examples 12 and 13). The fungal prototype is administered at an appropriate dose. SP-D ^{-/-} and SP-D ^{+/+} mice are used to test the effect of SP-D on susceptibility of mice to infection. SP-D ^{-/-} mice with or without SP-D protein is used to test SP-D as a therapy for infection. Clearance of infection is evaluated as in Examples 12 and 13 and as follows:

30 Fungal clearance is determined by purifying lung and spleen homogenates at 6, 24, and 48 hours after inoculation of the animals with infectious agent or infectious agent with SP-D. Bacterial clearance from the lungs is determined after varying SP-D concentrations appropriately. Quantitative cultures are also determined for the SP-D ^{+/-} mice to determine if 50% reduction in SP-D provides sufficient endogenous SP-D for bacterial or viral clearance.

Appropriate concentrations of mammalian SP-D are used in other mammals for treatment of pulmonary infections.

Pharmaceuticals that Regulate SP-D Levels

The importance of SP-D in normal function and development of the lung is clearly demonstrated by the SP-D (-/-) null mouse. Therefore, agents that regulate production, expression, or the action of SP-D are important future pharmaceuticals and experimental aids for identifying further such pharmaceuticals. Many techniques for identifying such agents would suggest themselves to one having ordinary skill in the art. Examples 15 and 16 outline a sample protocol for two of these techniques. Example 17 shows that IL-4 markedly increases SP-D levels *in vivo* and could thus be used to treat various pulmonary diseases with or without the addition of SP-D.

10

EXAMPLE 15

Proteins that interact with the SP-D promoter

A one-hybrid technique is set up using the SP-D promoter to identify proteins that up-regulate expression of SP-D. These proteins are then tested on the SP-D (-/-) mouse for efficacy in treating emphysema and other pulmonary diseases and infections as in Example 8.

15

EXAMPLE 16

Proteins that interact directly with the SP-D protein

A two-hybrid technique is set up to identify proteins that interact directly with the SP-D protein. These proteins are then be tested on the SP-D (-/-) mouse for efficacy in treating emphysema and other pulmonary diseases and infections as in Example 8.

20

EXAMPLE 17

IL-4 increases SP-D levels *in vivo*

Mice that express IL-4 in Clara cells (CCSP-IL-4) develop chronic airway inflammation and an alveolar proteinosis-like syndrome. In order to identify the role of IL-4 in surfactant homeostasis, we measured lipid and protein metabolism in the lungs of CCSP-IL-4 mice *in vivo*. Alveolar saturated phosphatidylcholine (Sat PC) pools were increased 6.5 fold and lung tissue Sat PC pools were increased 4.8 fold in the IL-4 transgenic mice (see Figure 15). SP-D was increased approximately 90 fold in the IL-4 mice compared to wild type mice and was associated with 2.8 fold increased SP-D mRNA (see Figure 15). The incorporation of palmitate and choline into Sat PC was increased about 2 fold in CCSP-IL-4 mice. Net clearance of Sat PC from the lungs of CCSP-IL-4 mice was 6 fold higher (60 mol/kg) in the IL-4 mice than in wild type mice (10.3 mol/kg). Expression of IL-4 in Clara cells increased surfactant lipid synthesis and clearance, establishing a new equilibrium with increased surfactant pools and an alveolar proteinosis associated with a selective increase in SP-D protein, demonstrating a previously unexpected effect of IL-4 in pulmonary surfactant homeostasis and the regulation of SP-D levels by IL-4.

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EXAMPLE 18**Diagnosis Using SP-D Protein or Sequence**

SP-D is important in normal lung function and development. SP-D (-/-) mice are a model for emphysema. This
5 then suggests that mutations in the gene or alleles of the gene for SP-D have a profound effect on pulmonary disease
susceptibility. Therefore, a method to identify mutations or alleles, and mutant protein identifies individuals at risk for
emphysema, pulmonary infections, and a number of other respiratory diseases. Example 18 and 19 are sample
protocols for these diagnostic techniques.

EXAMPLE 19**Diagnosis of Patients with Mutations in the SP-D gene**

Mutations in the SP-D gene are likely involved in the symptoms and etiology of emphysema. Therefore,
mutations are identified by sequence analysis of a statistically significant number of patients. These mutations are
used to produce a diagnostic test. Mutations in the SP-D gene are detected in the following ways: PCR analysis of
15 the SP-D gene using appropriate primers is performed. Resulting PCR fragments are analyzed by SSCP and sequenced
to determine mutation or allele. Alternatively, differential hybridization of genomic DNA or cDNA is used to detect
mutations.

EXAMPLE 20**Diagnosis of Patients With Mutant SP-D Protein**

20 Monoclonal or polyclonal antibodies which specifically recognize mutant SP-D protein or an allele of SP-D
associated with emphysema or other pulmonary diseases are produced. These antibodies are then used to set up an
enzyme-linked immunoassay for susceptibility to these pulmonary diseases. The antibodies of Example 20 can be
used for this assay.

25 Example 20 presents a protocol for the purification of polyclonal or further purification of monoclonal
antibodies using transgenic technology.

EXAMPLE 21**Purification of SP-D specific monoclonal and polyclonal antibodies**

30 The production of specific polyclonal antibodies with a high reactivity requires extensive purification of the
antigen of interest. We have developed several polyclonal antibodies using partially purified antigens for injection
which have resulted in antibodies which have a high titer with respect to the antigen of interest and are also reactive
to impurities. Solid phase tissue from transgenic mice have been used to remove nonspecific antibodies from these
antisera. Surfactant Protein-D (SP-D) was purified using a maltose column with manganese elution. The purified SP-D
35 was injected into New Zealand rabbits in incomplete Freund's adjuvant. The resulting antisera was tested against

whole lung lavage on a Western Blot, revealing binding to the SP-D and to other proteins. This antisera was reacted overnight with a solid phased lung homogenate from a null mutant mouse which does not produce any SP-D protein. The antisera was reacted against whole lung lavage after absorption showing reactivities only against SP-D. This antisera was also evaluated in immunohistochemistry experiments which demonstrated very low reactivities to lung sections from SP-D null mutant mice and very specific type II cell reactivities in normal control mice. This technique greatly enhances the ability to prepare highly specific antibodies with high titers and eliminates the need to use blocking agents when using absorbed antibodies.

These antibodies could be used for the diagnosis, purification, and further research into the SP-D protein.

10

EXAMPLE 22

SP-D inhibits viral infection

Previous results (Example 13) showed that SP-D has a role in the clearance of RSV from the lungs of mice. Therefore, it was of interest to see if SP-D had a similar role in the clearance of other viruses.

SP-D(-/-) mice were intratracheally infected with influenza A virus and separately with adenovirus. Viral titers and lung inflammation were assessed in SP-D (-/-) mice and wild type mice. Influenza A titers in lung homogenates were significantly increased in SP-D (-/-) compared to wild type mice 3 and 5 days after administration. Significantly increased numbers of inflammatory cells were found in BAL fluid from SP-D (-/-) mice with a greater percentage of PMNs compared to wild type mice, after Influenza A infection.

Therefore, SP-D deficient mice are susceptible to influenza A viral infection *in vivo* and developing markedly increased lung inflammatory responses to the virus and SP-D binds adenovirus *in vitro* and will likely play a role in clearance of adenovirus *in vivo* as well.

EXAMPLE 23

SP-D inhibits reactive lipid species

SP-D deficient surfactant has increased oxygen-lipid intermediates (toxic lipid reactants). Thus, SP-D inhibits reactive lipid species in the airspace and may have potential benefits for amelioration of reactive oxygen mediated disease, chemically induced lung injury, oxygen, ozone, chemotherapeutic agents and inflammatory diseases, reperfusion injury, drowning, transplantation, and rejection.

Reactive oxygen species were measured by the Lipid Hydroperoxide (LPO) assay kit (Caymen chemicals, Cat. No. 705002). Surfactant was isolated from SP-D knockout and wildtype mice by lung lavage and the lipid peroxidation products measured using redox reactions with ferrous ions. No lipid peroxides were detected in surfactant from wild type mice (n=4) but were readily detected in lavage fluid from SP-D (-/-) mice, 0.896 ± 0.305 ng of lipid peroxidation product /mg of phospholipid (n=4).

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EXAMPLE 24**SP-D inhibits viral infection**

Previous results (Example 13) showed that SP-D has a role in the clearance of RSV from the lungs of mice. Therefore, it was of interest to see if SP-D had a similar role in the clearance of other viruses.

5 SP-D(-/-) mice were intratracheally infected with influenza A virus and separately with adenovirus. Viral titers and lung inflammation were assessed in SP-D (-/-) mice and wild type mice. Influenza A titers in lung homogenates were significantly increased in SP-D (-/-) compared to wild type mice 3 and 5 days after administration. Significantly increased numbers of inflammatory cells were found in BAL fluid from SP-D (-/-) mice with a greater percentage of PMNs compared to wild type mice, after Influenza A infection.

10 Therefore, SP-D deficient mice are susceptible to influenza A viral infection *in vivo* and developing markedly increased lung inflammatory responses to the virus and SP-D binds adenovirus *in vitro* and will likely play a role in clearance of adenovirus *in vivo* as well.

WHAT IS CLAIMED IS:

1. A non-human mammal having an SP-D (-/-) null phenotype.
2. The non-human mammal of claim 1 wherein said non-human mammal is a mouse.
- 5 3. The non-human mammalian model of claim 2 wherein said mouse has a deletion of sequences from exon 2 of the SP-D gene including the initiating methionine and translation initiation sequences.
4. A method for the prevention and treatment of pulmonary disease comprising: introducing mammalian SP-D protein, or vectors expressing the mammalian SP-D protein into a human in an amount effective to reduce the symptoms of or prevent pulmonary disease.
- 10 5. The method of Claim 4 wherein the pulmonary disease is emphysema.
6. The method of Claim 4 wherein said SP-D protein is administered intra-tracheally.
7. The method of Claim 4 wherein said SP-D protein is expressed from an adenoviral vector.
8. The method of Claim 4 wherein said adenoviral vector is introduced via aerosolization.
9. The method of Claim 4 wherein said adenoviral vector is the adenoviral vector Ad-rSPD deposited
15 under the Accession No.
10. A pharmaceutical composition effective in treating pulmonary disease in mammals comprising: SP-D protein in admixture with a pharmaceutically acceptable excipient.
11. A biologically active agent for treating pulmonary disease in mammals comprising an agent that up-regulates expression of SP-D.
- 20 12. The biologically active agent of Claim 11 wherein said agent is IL-4.
13. A biologically active agent for treating pulmonary disease in mammals comprising a vector expressing SP-D.
14. The biologically active agent of Claim 13 wherein said vector is an adenovirus.
15. The biologically active agent of Claim 14 wherein said adenovirus vector is the Ad-rSPD of the
25 present invention.
16. A biologically active agent for treating pulmonary disease in mammals comprising an agent that interacts with the SP-D protein.
17. A method for diagnosing susceptibility to pulmonary disease comprising the steps of:
identifying a mutation in the SP-D gene which results in deficient SP-D
30 identifying said mutation in a test subject.
18. The method of diagnosing susceptibility to pulmonary disease of Claim 17 wherein said mutation is identified by PCR.
19. The method of diagnosing susceptibility to pulmonary disease of Claim 17 wherein said mutation is identified by hybridization.

20. The method of diagnosing susceptibility to pulmonary disease of Claim 17 wherein said mutation is identified by ELISA.

21. A method of identifying pharmaceutical agents useful in treatment of pulmonary disease comprising the steps of:

5 allowing the mammal of claim 1 to develop a pulmonary disease
administering a pharmaceutical agent to said mammal, and
identifying said agent as effective if said pulmonary disease improves.

22. A method of purifying SP-D antibodies comprising:
reaction of SP-D antibodies with a solid phase lung homogenate from any mouse with does not
10 produce SP-D protein.

23. The method of Claim 22 wherein said mouse is an SP-D null mouse.

24. A method of purifying SP-D antibodies comprising:
reaction of SP-D antibodies with a solid phase lung homogenate from the SP-D null mouse of
claim.

15 25. A method for the prevention and treatment of pulmonary disease comprising:
introducing mammalian SP-D protein, or vectors expressing the mammalian SP-D protein into a
human in an amount effective to reduce the symptoms of or prevent pulmonary disease, wherein the pulmonary
disease is selected from the group consisting of: reactive oxygen-mediated disease, chemically induced lung injury,
injury due to oxygen radicals, injury due to ozone, injury due to chemotherapeutic agents, inflammatory and infectious
20 diseases, reperfusion injury, drowning, transplantation, and rejection.

26. A method for the prevention and treatment of viral disease comprising:
introducing mammalian SP-D protein, or vectors expressing the mammalian SP-D protein into a
human in an amount effective to reduce the number of viruses or symptoms of the viral disease.

27. The method of Claim 26 wherein the viral disease is caused by a virus selected from the group
25 consisting of: Adenovirus, RSV, and Influenza virus.

28. A method of inhibition of metalloproteinase activity and reactive oxygen species in the lungs,
comprising, administering SP-D to the lungs in an amount effective to inhibit metalloproteinase activity and reactive
oxygen species.

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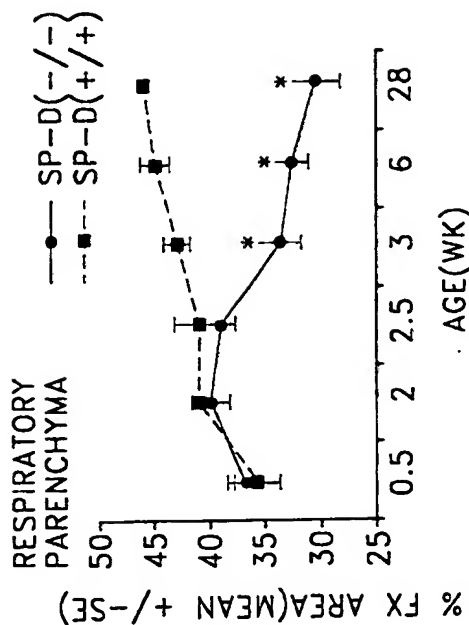


FIG. 1A

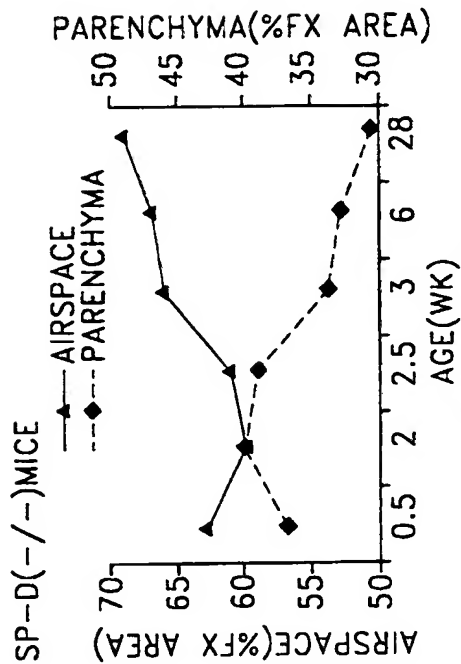


FIG. 1B

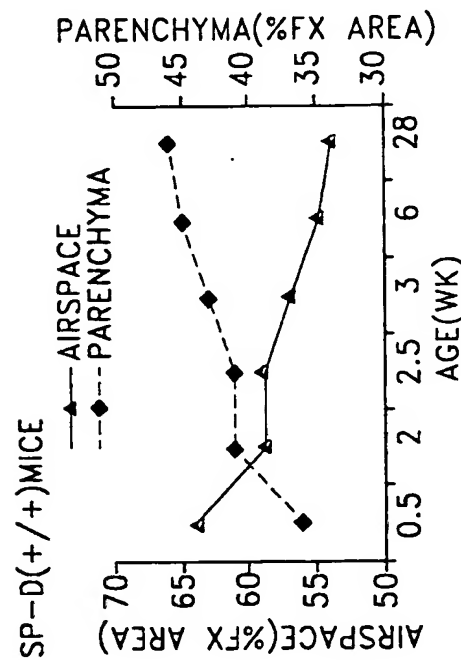
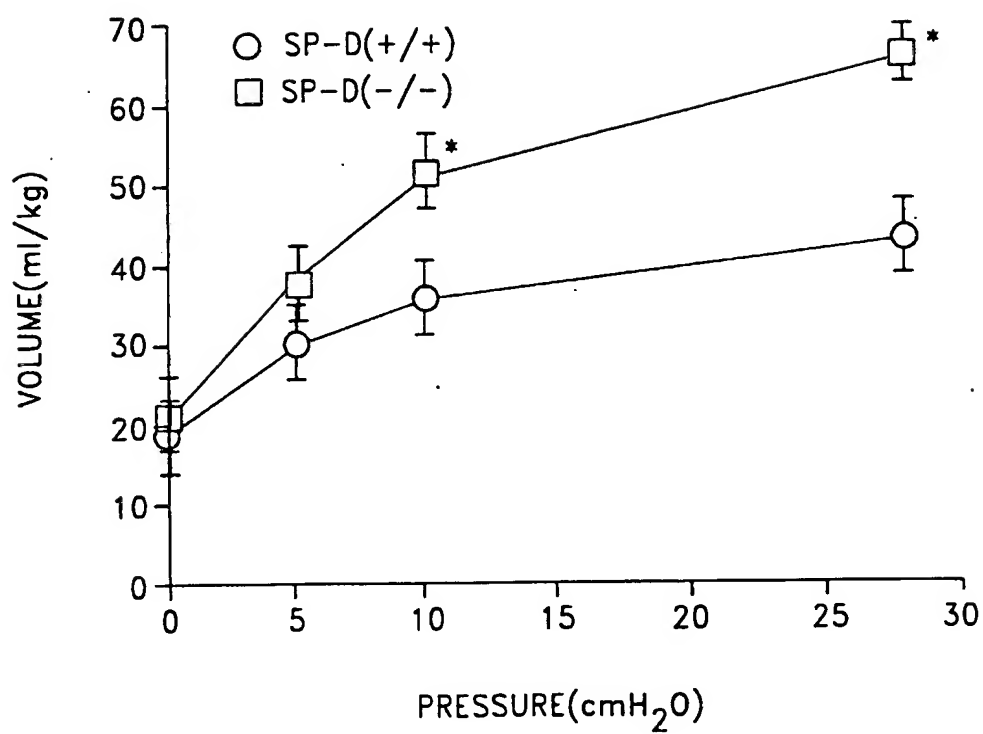
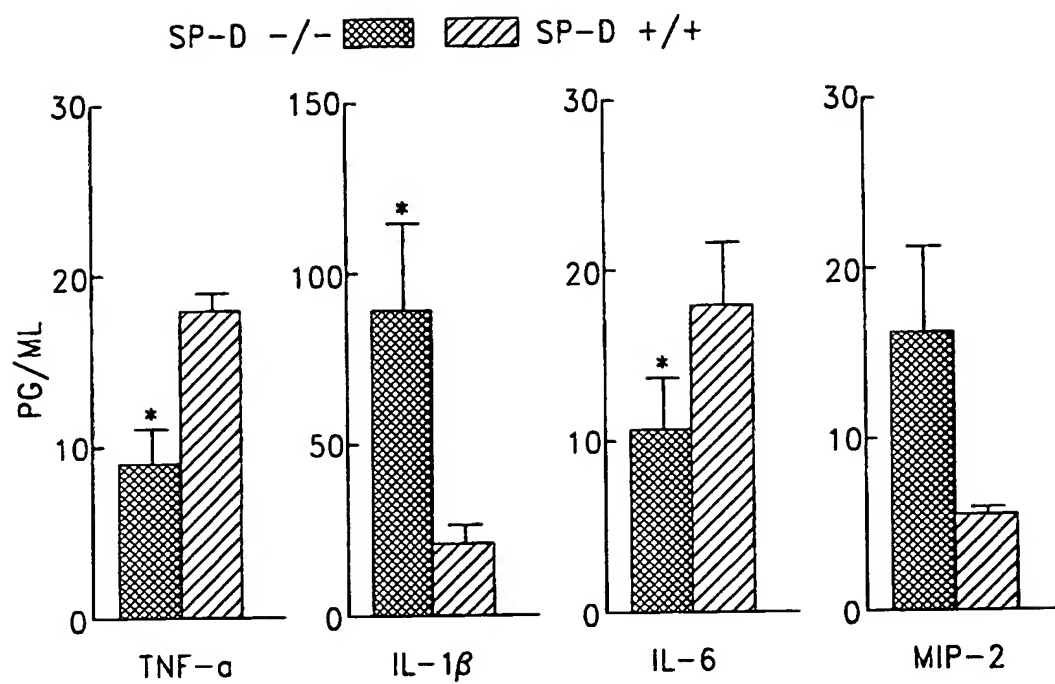


FIG. 1C

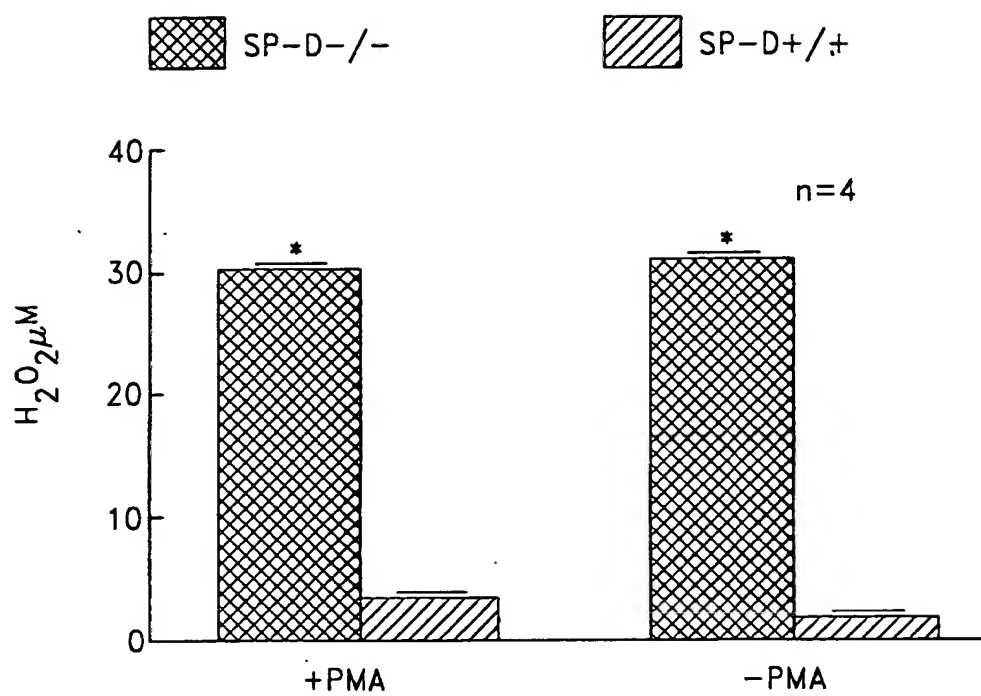
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*FIG.2*

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**FIG.3**

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*FIG. 4*

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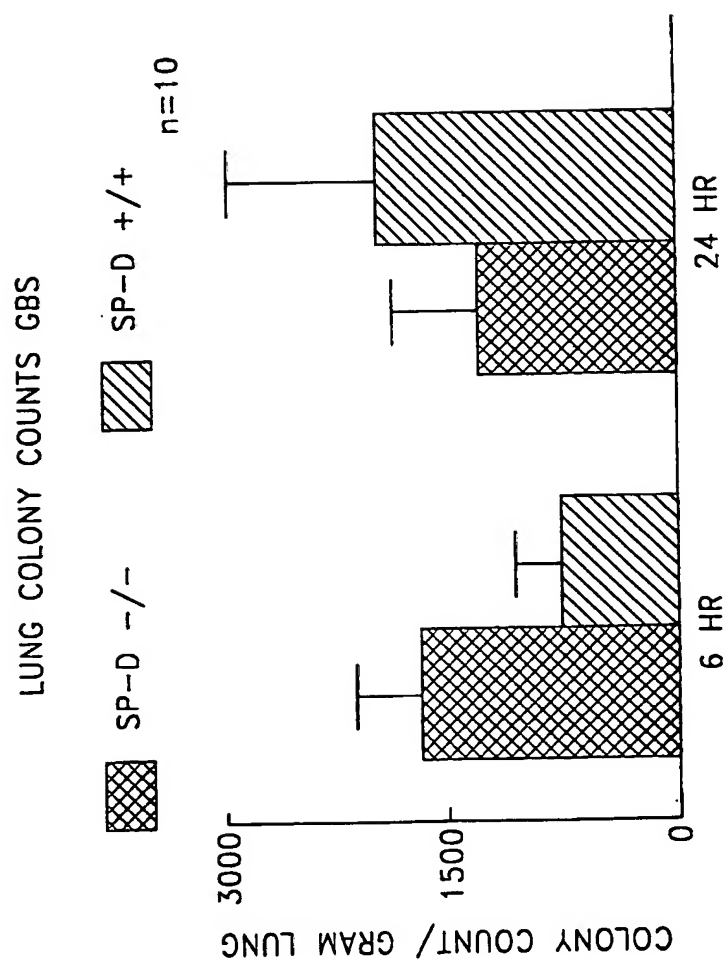


FIG.5

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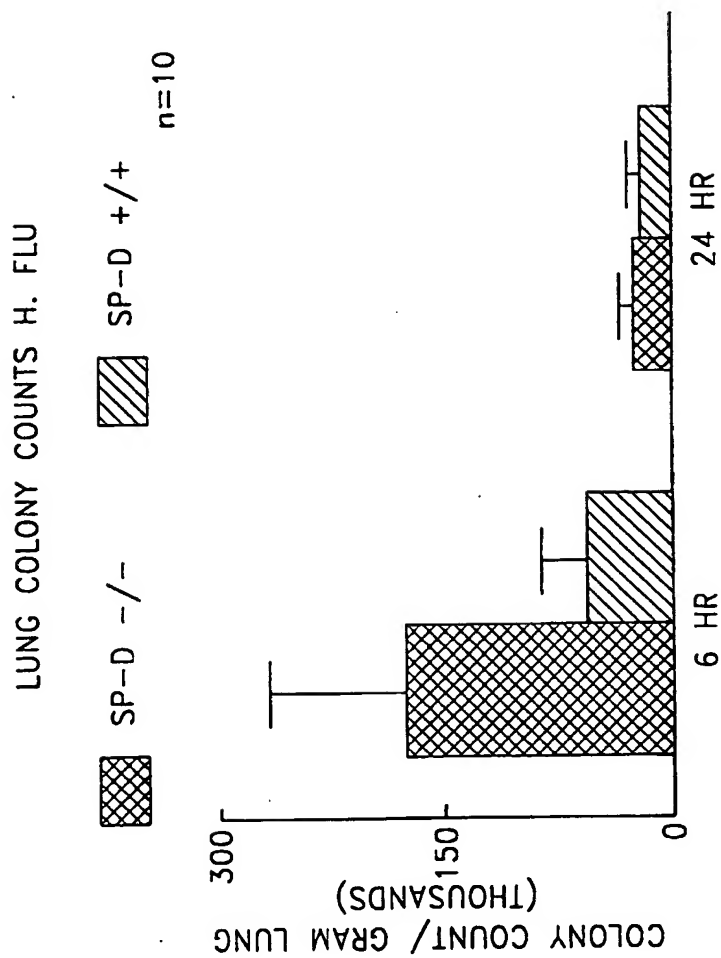
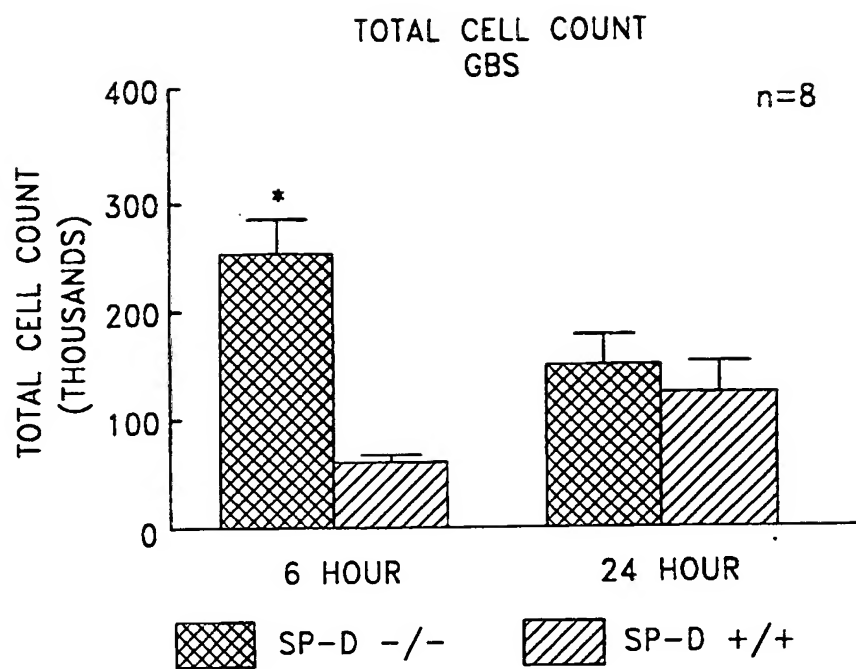
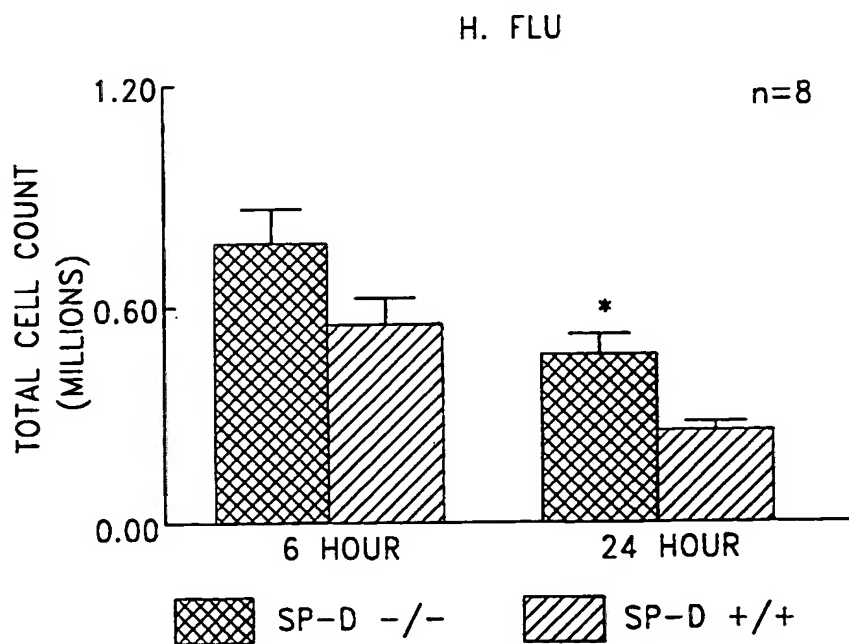
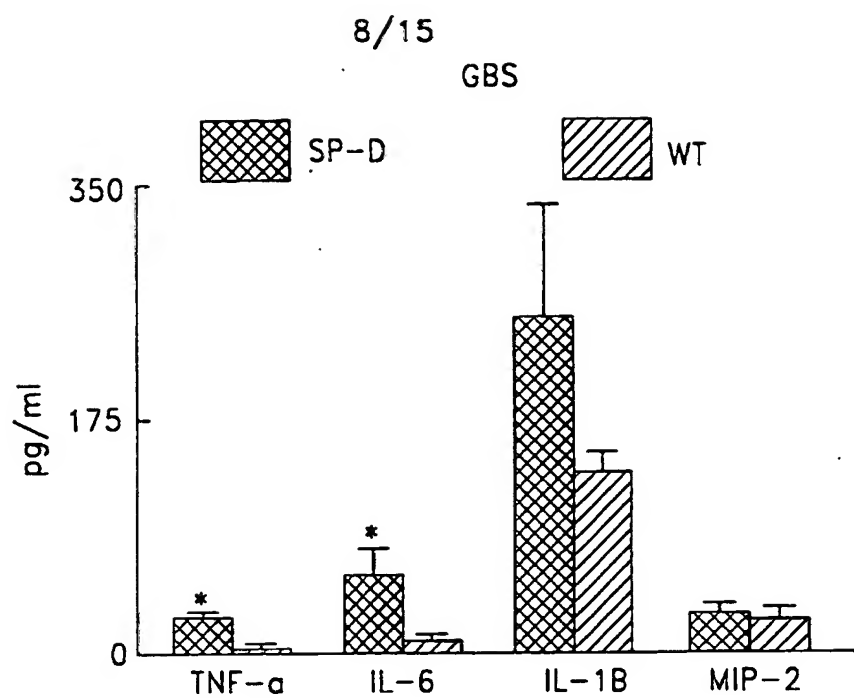
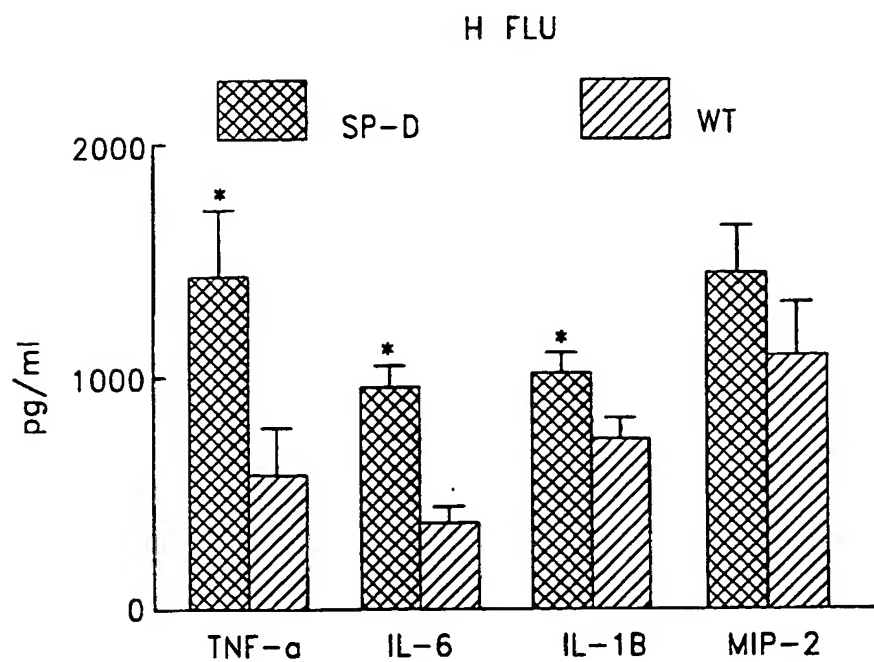


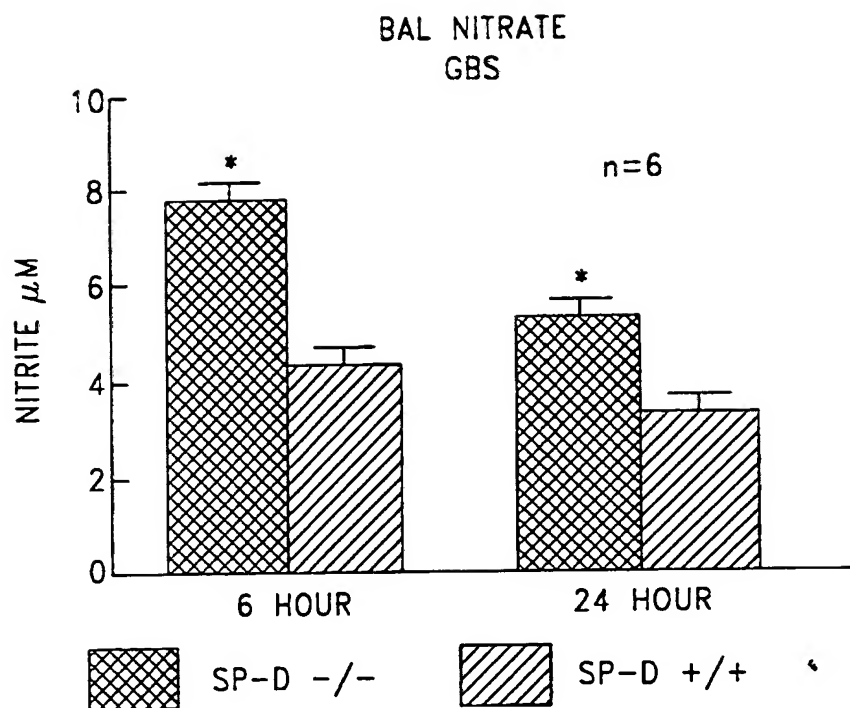
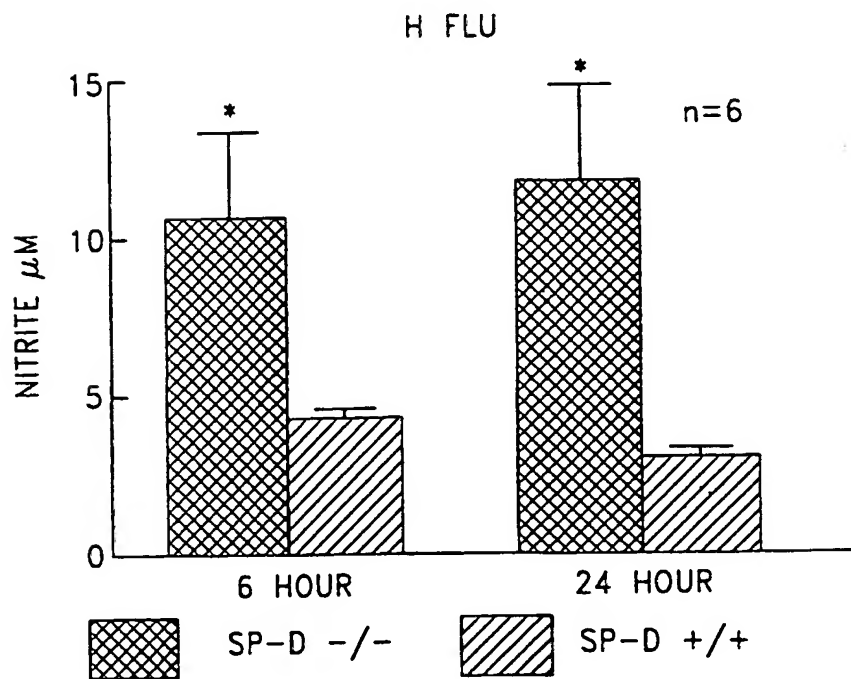
FIG.6

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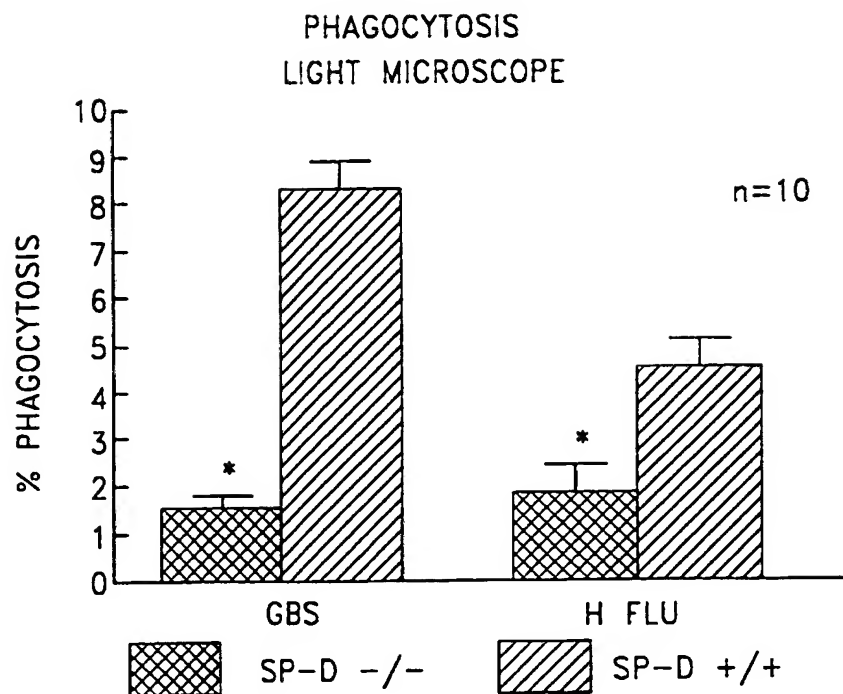
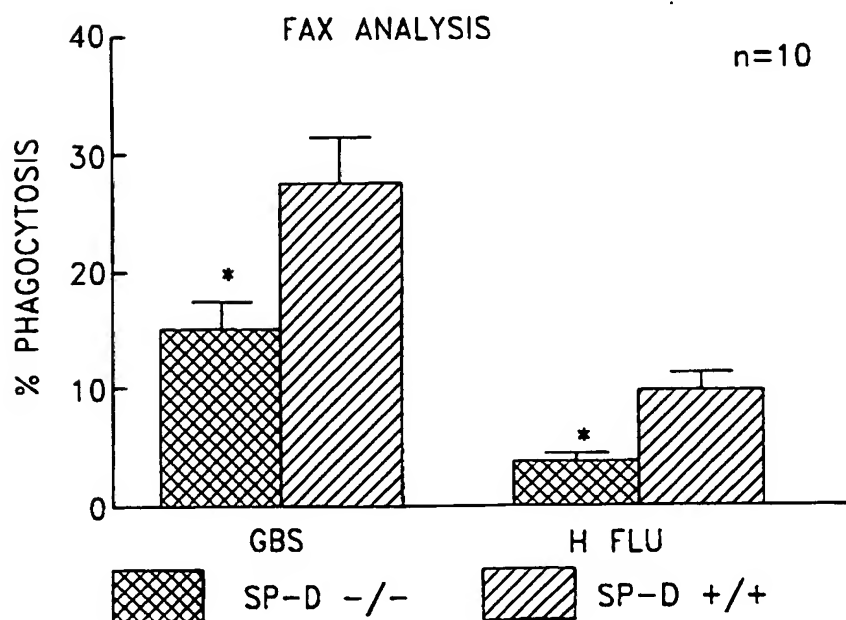
*FIG. 7A**FIG. 7B*

*FIG. 8A**FIG. 8B*

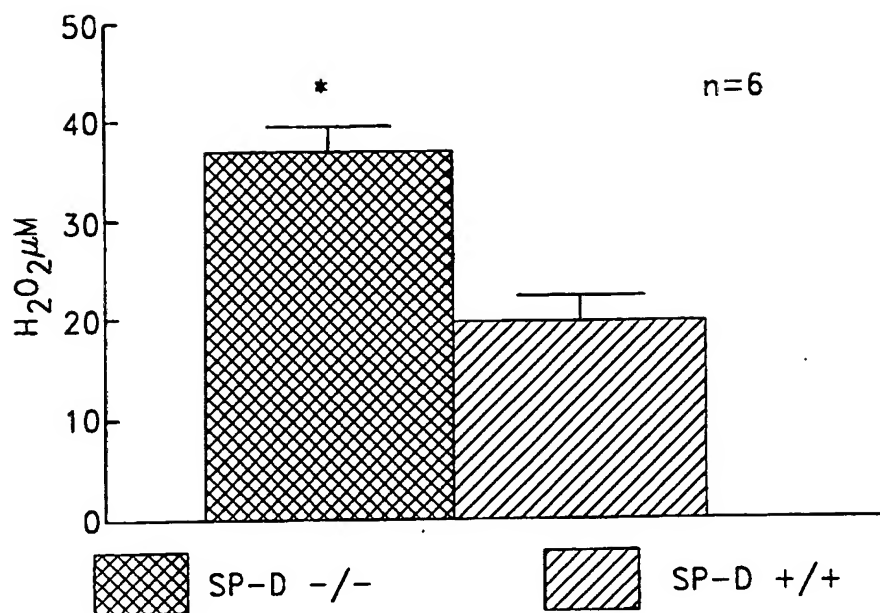
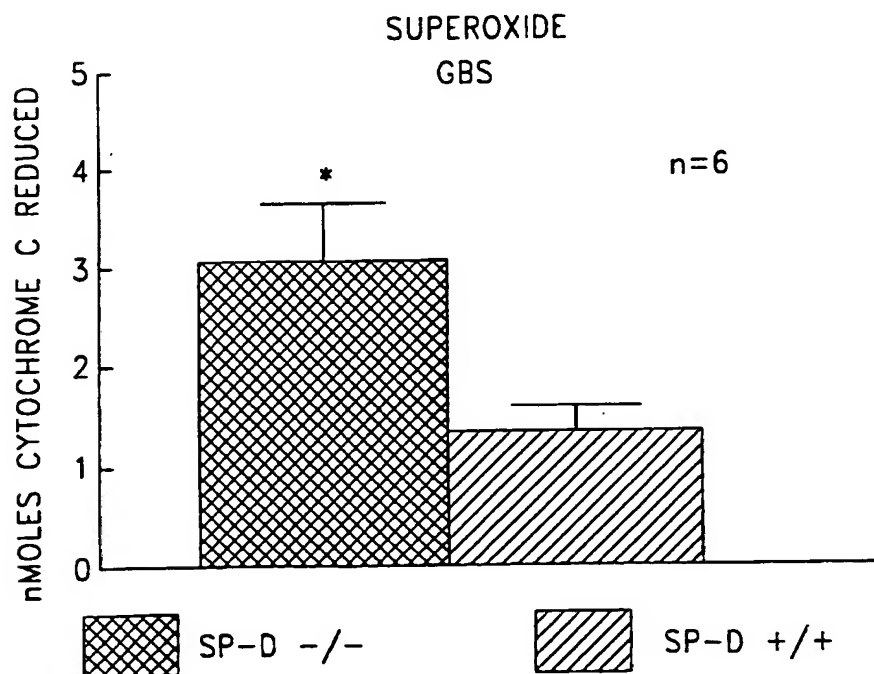
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**FIG. 9A****FIG. 9B**

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*FIG. 10A**FIG. 10B*

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HYDROGEN PEROXIDE
GBS*FIG. 11A**FIG. 11B*

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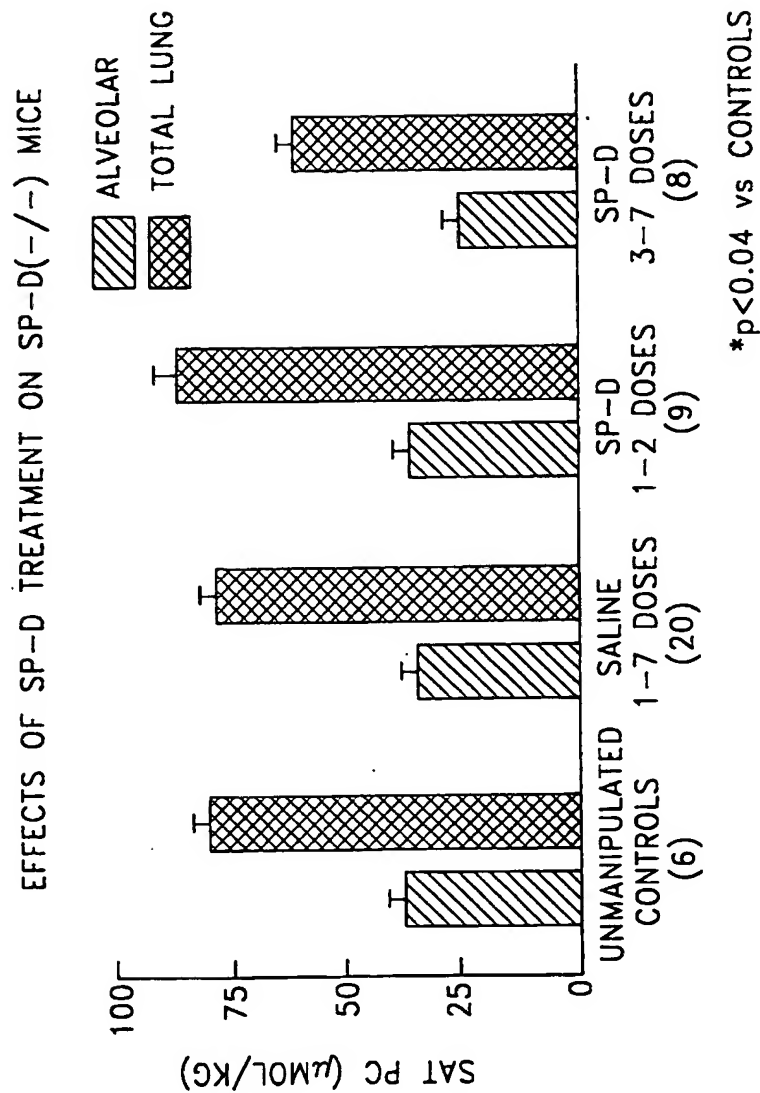
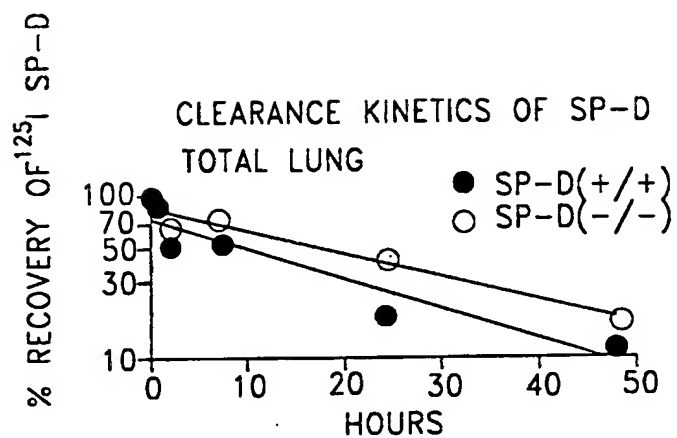
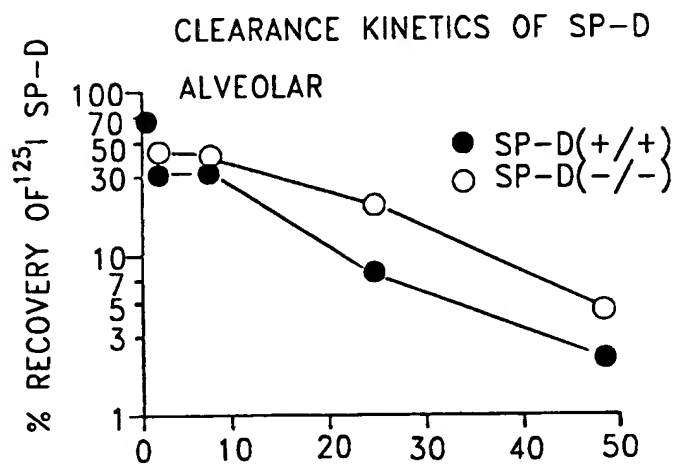


FIG. 12

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*FIG. 13A**FIG. 13B*

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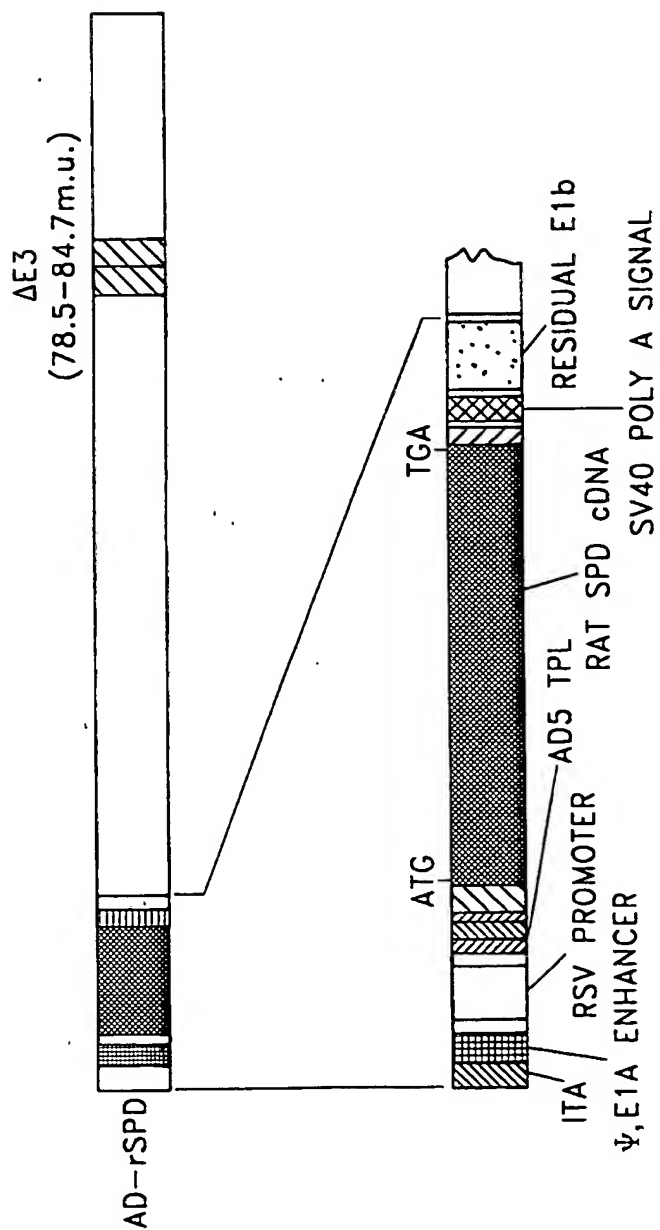
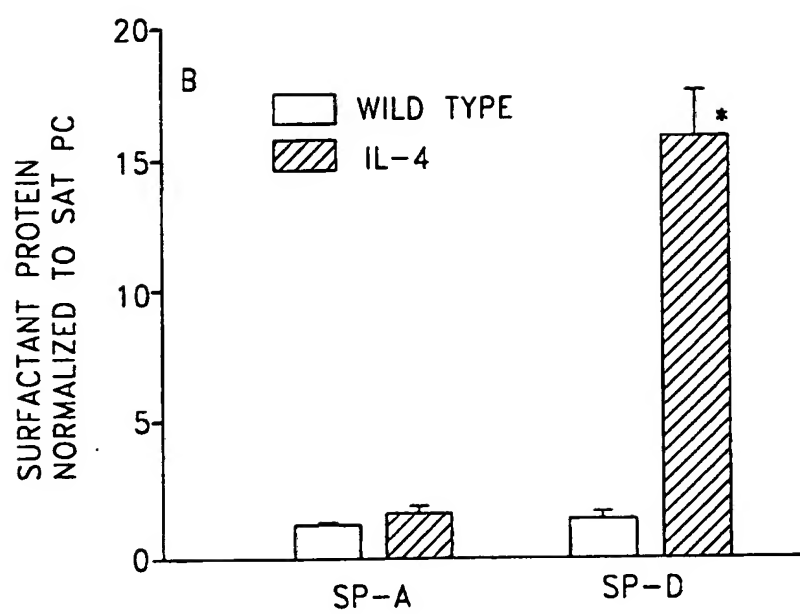


FIG. 14

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*FIG. 15*

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KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,
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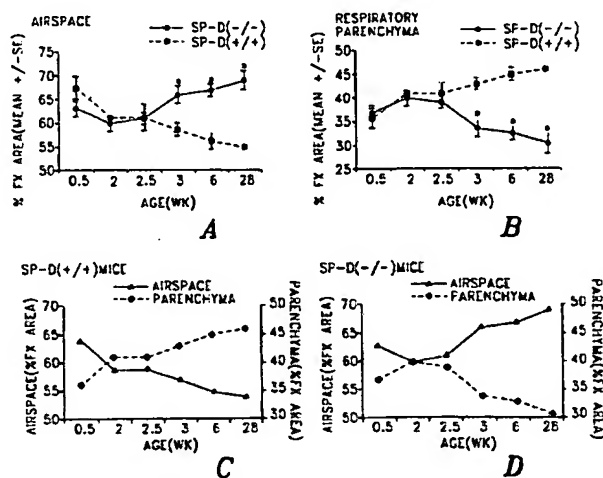
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[Continued on next page]

(54) Title: SURFACTANT PROTEIN D FOR THE PREVENTION AND DIAGNOSIS OF PULMONARY EMPHYSEMA



(57) Abstract: Surfactant protein D (SP-D) is a 43-kDa member of the collectin family of collagenous lectin domain-containing proteins that is expressed in epithelial cells of the lung. The SP-D gene was targeted by homologous recombination in embryonic stem cells that were used to produce SP-D^{-/-} mice. The SP-D^{-/-} deficiency caused inflammation, increased oxidant production by isolated alveolar macrophages, abnormal surfactant structure and levels, and decreased SP-A expression. Therefore, disclosed is the SP-D^{-/-} mouse as an excellent model for emphysema. Also included are models for testing emphysema therapies in the mouse model, methods for using SP-D protein or DNA as a treatment for emphysema and pulmonary infections, and diagnosis.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

ational Application No
PCT/US 01/13747

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/00 A01K67/027 C07K14/785 A61K49/00 C07K16/18
G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC-7 A01K - C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOTAS C, ET AL.: "Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D." PROC NATL ACAD SCI U S A. 95(20):11869-74.. 29 September 1998 (1998-09-29), XP001059873 the whole document	1-3
P,X	WO 00 23569 A (CHILDRENS HOSP MEDICAL CENTER ;WHITSETT JEFFREY A (US)) 27 April 2000 (2000-04-27) the whole document	1-3

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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8 document member of the same patent family

Date of the actual completion of the international search

11 February 2002

Date of mailing of the international search report

07.06.02

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Authorized officer

CHAM BONNET, F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/13747

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: -- 9 24
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 21

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 01/13747

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3, 21

A non human mammal having an SP-D (-/-) null phenotype; a method for identifying pharmaceutical agents useful in treatment of pulmonary disease using said mammal;

2. Claims: 4-10, 13-15, 25 and partially 26-27

A method for the prevention and treatment of pulmonary disease comprising : introducing mammalian SP-D protein or vectors expressing the mammalian SP-D protein into a human in an amount effective to reduce the symptoms of or prevent pulmonary disease; a pharmaceutical composition effective in treating pulmonary disease in mammals comprising SP-D protein in admixture with a pharmaceutically acceptable excipient; a biologically active agent for treating pulmonary disease in mammals comprising an agent a vector expressing SP-D;

3. Claims: 11, 12 and partially 16

A biologically active agent for treating pulmonary disease in mammals comprising an agent that upregulates expression of SP-D;

4. Claim : Partially 16

A biologically active agent for treating pulmonary disease in mammals comprising an agent that interacts with the SP-D protein, as far as not covered by the previous subject;

5. Claims: 17-20

A method for diagnosing susceptibility to pulmonary disease comprising the steps of identifying a mutation in the SP-D gene which results in deficient SP-D identifying said mutation in a test subject;

6. Claims: 22-24

A method of purifying SP-D antibodies comprising: reaction of SP-D antibodies with a solid phase lung homogenate from any mouse with does not produce SP-D protein.

7. Claims: Partially 26-27

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 01/13747

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method for the prevention and treatment of viral disease comprising : introducing mammalian SP-D protein or vectors expressing the mammalian SP-D protein into a human in an amount effective to reduce the number of virus or symptoms of the viral disease, as far as not covered by a previous subject;

8. Claim : 28

A method of inhibition of metalloproteinase activity and reactive oxygen species in the lungs comprising administering SP-D to the lungs in an amount effective to inhibit metalloproteinase activity and reactive oxygen species, as far as not covered by a previous subject.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 01/13747

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Claims 4 to 9 and 25 to 27, as well as 28 as far as it would concern a in vivo therapy, are directed to a method of treatment of the human/animal body. Any eventual further paid search could only be carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 9 24

In case of further search would be paid :

Claim 9 is related to a vector deposited under a missing Accession No.

Claim 24 concerns SP-D null mouse from a previous claim but the number of this claim referred to is missing. Either claim 2 or 3 could both be possible.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/13747

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0023569	A	27-04-2000	AU 1598000 A	08-05-2000
			BR 9914645 A	05-02-2002
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			WO 0023569 A1	27-04-2000
